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  * * * * * * * * * * * * * STN  Columbus  * * * * * * * * * * * * * * *

FILE 'HOME' ENTERED AT 16:12:42 ON 01 JUL 2002

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COST IN U.S. DOLLARS                                    SINCE FILE      TOTAL
                                                           ENTRY    SESSION
FULL ESTIMATED COST                                         0.21       0.21

FILES 'MEDLINE, SCISEARCH, LIFESCI, BIOTECHDS, BIOSIS, EMBASE, HCAPLUS, NTIS,
       ESBIOBASE, BIOTECHNO, WPIDS' ENTERED AT 16:12:55 ON 01 JUL 2002
ALL COPYRIGHTS AND RESTRICTIONS APPLY.  SEE HELP USAGETERMS FOR DETAILS.

11 FILES IN THE FILE LIST

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FILE 'MEDLINE'
          1368 CHITINASE#
            52 CHITOTRIOSIDASE#
L1        1403 CHITINASE# OR CHITOTRIOSIDASE#

FILE 'SCISEARCH'
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L2        2885 CHITINASE# OR CHITOTRIOSIDASE#

FILE 'LIFESCI'
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L3        1352 CHITINASE# OR CHITOTRIOSIDASE#

FILE 'BIOTECHDS'
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             2 CHITOTRIOSIDASE#
L4         822 CHITINASE# OR CHITOTRIOSIDASE#

FILE 'BIOSIS'
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            68 CHITOTRIOSIDASE#
L5        3387 CHITINASE# OR CHITOTRIOSIDASE#

FILE 'EMBASE'
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FILE 'HCAPLUS'
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L7        4004 CHITINASE# OR CHITOTRIOSIDASE#

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FILE 'LIFESCI'
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L15          20 L3 (5A)HUMAN

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FILE 'EMBASE'
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L18          30 L6 (5A)HUMAN

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TOTAL FOR ALL FILES
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SEARCH ENDED BY USER

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TOTAL FOR ALL FILES
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L36          0 L13 AND PY=<1996

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COST IN U.S. DOLLARS                                SINCE FILE      TOTAL
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FULL ESTIMATED COST                                     7.60      22.20

STN INTERNATIONAL LOGOFF AT 16:27:01 ON 01 JUL 2002
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1	L1	1144	chitinase$1 or chitotriosidase$1	USPAT; US-PGPUB	2002/07/01 16:04
2	L2	16	1 near4 human	USPAT; US-PGPUB	2002/07/01 16:05

US-PAT-NO: 6399571
DOCUMENT-IDENTIFIER: US 6399571 B1
TITLE: Chitinase chitin-binding fragments
DATE-ISSUED: June 4, 2002
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Gray; Patrick W.	Seattle	WA	N/A	N/A
Tjoelker; Larry W.	Kirkland	WA	N/A	N/A

US-CL-CURRENT: 514/12,424/94.61 ,435/209 ,530/350
ABSTRACT:

The present invention provides chitin-binding fragments of **human chitinase,** fragment analogs, purified and isolated polynucleotide sequences encoding such fragments and analogs, and materials and methods for the recombinant production of **human chitinase** fragment products which are expected to be useful as in products for detecting chitin, binding chitin, and treating fungal infections or for development of products useful for treating the same.
24 Claims, 0 Drawing figures
Exemplary Claim Number: 1

DATE FILED: March 12, 1999

---------- KWIC ---------

ABPL:
The present invention provides chitin-binding fragments of **human chitinase,** fragment analogs, purified and isolated polynucleotide sequences encoding such fragments and analogs, and materials and methods for the recombinant production of **human chitinase** fragment products which are expected to be useful as in products for detecting chitin, binding chitin, and treating fungal infections or for development of products useful for treating the same.

BSPR:
The present invention relates generally to materials comprising chitin-binding fragments of **human chitinase** enzyme and analogs of the fragments. More particularly, the invention relates to novel purified and isolated polynucleotides encoding such fragment products, to the chitinase fragment products encoded by such polynucleotides, to materials and methods for the recombinant production of such chitinase fragment products and to therapeutic and diagnostic uses of such chitinase fragment products.

BSPR:
Escott et al., Infect. Immun., 63:4770-4773 (1995) demonstrated **chitinase enzymatic activity in human** leukocytes and in human serum. Overdijk et al., Glycobiology, 4:797-803 (1994) described isolation of a chitinase (4-methylumbelliferyl-tetra-N-acetylchitotetraoside hydrolase) from human serum and rat liver. Renkema et al., J. Biol. Chem., 270:2198-2202 (February 1995) prepared a **human chitotriosidase** from the spleen of a Gaucher disease patient. Their preparation exhibited chitinase activity and the article reports a small

amount of amino acid sequence of the protein component of the preparation (22 amino terminal residues and 21 residues of a tryptic fragment). The function of **human chitinase** is also unknown, but a relationship with the pathophysiology of Gaucher disease is proposed in the article. A later publication by the same group [Boot et al., J. Biol. Chem., 270(44):26252-26256 (November 1995)] describes the cloning of a human macrophage cDNA encoding a product that exhibits chitinase activity. The partial amino acid sequence reported by the group in their February 1995 article matches portions of the deduced amino acid sequence of the human macrophage cDNA product. See also International Patent Publication No. WO 96/40940, which reports two distinct **human chitotriosidase** cDNAs encoding a 50 kD and a 39kD product, both of which were fully enzymatically active. Renkema et al., Eur. J. Biochem., 244:279-285 (1997) reported that **human chitinase** is initially produced in macrophages as a 50 kD protein that is in part processed into a 39 kD form that accumulates in lysozymes, and also reported that alternative splicing generates a distinct **human chitinase** mRNA species encoding a 40 kD chitinase. Both the 39 kD and 40 kD isoforms appeared to be C-terminally truncated and displayed full chitinase enzymatic activity but bound chitin poorly.

BSPR:
The present invention provides novel purified and isolated polynucleotides (i.e., DNA and RNA, both sense and antisense strands) encoding **human chitinase** fragments and analogs thereof having chitin-binding activity but lacking chitinase enzymatic activity; methods for the recombinant production of such fragment products; purified and isolated **human chitinase** polypeptide fragment products; pharmaceutical compositions comprising such fragment products; and diagnostic or therapeutic agents conjugated to such fragment products thereof. Such fragment products and diagnostic or therapeutic agents conjugated thereto are expected to be useful for detecting chitin, binding chitin, and treating fungal infections or for development of products useful for treating fungal infections.

BSPR:
The nucleotide sequence of two human cDNAs encoding presumed allelic variants of **human chitinase,** and including noncoding 5' and 3' sequences, are set forth in SEQ ID NO: 1 and SEQ ID NO: 3. The **human chitinase** coding region corresponds to nucleotides 2 to 1399 of SEQ ID NO: 1 or nucleotides 27 to 1424 of SEQ ID NO: 3, and the putative coding sequence of the mature, secreted **human chitinase** protein without its signal sequence corresponds to nucleotides 65 to 1399 of SEQ ID NO: 1, or nucleotides 90 to 1424 of SEQ ID NO: 3. The amino acid sequences of the polypeptides encoded by the DNA of SEQ ID NOS: 1 and 3 are set forth in SEQ ID NO:2 and SEQ ID NO: 4, respectively. Twenty-one amino-terminal amino acids (positions -21 to -1 of SEQ ID NOS: 2 and 4) comprise a signal peptide that is cleaved to yield the mature **human chitinase** protein (positions 1 to 445 of SEQ ID NOS: 2 and 4). It has been determined that the seventy-two C-terminal residues of **human chitinase** are not critical to chitinase enzymatic activity. Example 5 below illustrates production of an N-terminal fragment that lacks the seventy-two C-terminal residues of **human chitinase**; the introduction of a stop codon after the codon for amino acid 373 resulted in a recombinant chitinase fragment of about 39 kDa that retained similar specific chitinase enzymatic activity when compared with full length recombinant **human chitinase**. The cloning of **human chitinase** cDNA and

expression thereof, and the biological activities of recombinant **human chitinase** are described in detail in U.S. application Ser. No. 08/877,599 filed Jun. 16, 1997, which is a continuation-in-part of U.S. application Ser. No. 08/663,618 filed Jun. 14, 1996, both of which are incorporated herein by reference in their entirety.

BSPR:
The present invention is based on the unexpected discovery that substantially all of the chitin-binding activity of **human chitinase** is contained within the 99 C-terminal amino acid residues of the 445 amino acid enzyme. Specifically provided by the present invention are chitin-binding, chitinase-inactive polypeptide products. Preferred chitinase fragment products comprise a chitin-binding fragment within the 54 C-terminal amino acids of **human chitinase,** including a fragment consisting of about the 99 C-terminal amino acids of **human chitinase** (about residues 347 through 445 of SEQ ID NO: 2), a fragment consisting of about the 72 C-terminal amino acids of **human chitinase** (about residues 374 through 445 of SEQ ID NO: 2), a fragment consisting of about the 54 C-terminal amino acids of **human chitinase** (about residues 392 through 445 of SEQ ID NO: 2), and a fragment consisting of about the 49 C-terminal amino acids of **human chitinase** (about residues 397 through 445 of SEQ ID NO: 2). Also provided by the invention are purified, isolated polynucleotides including DNA encoding such polypeptide fragments; vectors comprising such DNAs, particularly expression vectors wherein the DNA is operatively linked to an expression control DNA sequence; host cells stably transformed or transfected with such DNAs in a manner allowing the expression in said host cell of **human chitinase** fragment products; a method for producing **human chitinase** polypeptide fragment products comprising culturing such host cells in a nutrient medium and isolating such polypeptides from said host cell or said nutrient medium; purified, isolated polypeptides produced by this method; fusion proteins comprising such polypeptides fused to a heterologous peptide or polypeptide, including an enzyme such as secreted alkaline phosphatase (SEAP); compositions comprising such human polypeptide fragment products; compositions comprising a **human chitinase** polypeptide fragment product conjugated to an anti-fungal agent and methods of treating fungal infection by administering such compositions, optionally with co-administration of additional non-chitinase anti-fungal agents; compositions comprising a chitinase polypeptide fragment product conjugated to a detectable label (including radioisotopes, fluorophores, dyes, electron-dense compounds and enzymes), methods for using such compositions to determine the presence or amount of chitin in a sample, comprising the steps of: (a) contacting the sample with a **human chitinase** polypeptide fragment product conjugated to a detectable label, and (b) determining the amount of labelled fragment product bound to chitin, and corresponding kits for diagnosing the presence of chitin in a sample; monoclonal antibodies that specifically bind a chitin-binding, **chitinase-inactive fragment of human chitinase,** including antibodies that specifically bind to an epitope within the 54 C-terminal amino acids of **human chitinase** as set forth in SEQ ID NO:2; and preferred monoclonal antibodies 243Q and 243M, and antibodies that compete with or bind to the same epitope as 243Q and 243M.

BSPR:
Chitinase polypeptide fragment products of the invention include fragments of

human chitinase or allelic variants thereof that substantially retain chitin-binding activity without retaining substantial chitinase enzymatic activity, analogs of such fragments, and fusion proteins comprising such fragments or analogs. Chitinase polypeptide fragment products are useful in therapeutic and diagnostic applications as described below.

BSPR:
Among the "chitin-binding domain" fragments contemplated by the invention are those represented by amino acid residues X through Y of SEQ ID NO: 2, wherein X is a consecutive integer from 347 through 397 and Y is 445, and portions thereof that retain chitin-binding activity. One preferred fragment consists of the ninety-nine C-terminal amino acids of **human chitinase** (residues 347 through 445 of SEQ ID NO: 2); this fragment has been shown in Example 7 below to retain 80% of the chitin-binding activity of the mature chitinase. Yet other preferred fragments are the fifty-four C-terminal amino acids of **human chitinase** (residues 392 through 445 of SEQ ID NO: 2), and the 49 C-terminal amino acids of **human chitinase** (residues 397 through 445 of SEQ ID NO:2), which have also been shown in Example 7 to retain chitin-binding activity. As illustrated in Example 7, a fusion protein containing the 99 C-terminal amino acids of **human chitinase** was shown to contain the chitin-binding domain of the protein. The boundaries of the chitin-binding domain were further defined by N-terminal and C-terminal truncation of this 99 amino acid region and determination of the chitin binding activity of fusion proteins comprising these truncates. These truncates included those with an N-terminus commencing at amino acid residue 347, 374, 392, 395, 397, 400 or 409 and with a C-terminus at amino acid residue 431, 443 or 445.

BSPR:
Preferred DNA sequences of the invention include genomic and cDNA sequences as well as wholly or partially chemically synthesized DNA sequences encoding chitin-binding fragments of **human chitinase** without chitinase enzymatic activity, analogs thereof, and fusion proteins comprising such fragments or analogs. Among the nucleotide sequences contemplated by the invention are those encoding the amino acid sequences of positions X through Y of SEQ ID NO: 2, wherein X is a consecutive integer from 347 through 392 and Y is 445. Nucleotides 1238 through 1399 of SEQ ID NO: 1 (encoding residues 392 through 445 of SEQ ID NO: 2) are a particularly preferred DNA sequence of the invention. This DNA sequence and other DNA sequences which hybridize to the noncoding strand thereof under standard stringent conditions or which would hybridize but for the redundancy of the genetic code, and which encode chitin-binding fragments of a chitinase, are also contemplated by the invention. Exemplary stringent hybridization conditions are as follows: hybridization at 42.degree. C. in 50% formamide and washing at 60.degree. C. in 0.1.times.SSC, 0.1% SDS. It is understood by those of skill in the art that variation in these conditions occurs based on the length and GC nucleotide base content of the sequences to be hybridized. Formulas standard in the art are appropriate for determining exact hybridization conditions. See Sambrook et al., 9.47-9.51 in Molecular Cloning, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989).

BSPR:
Among the uses for the polynucleotides of the present invention are use as a hybridization probe, to identify and isolate non-human genomic DNA and cDNA

encoding chitin-binding regions of proteins homologous to **human chitinase**; and to identify those cells which express chitin-binding portions of such proteins and the biological conditions under which such proteins are expressed.

BSPR:
In another aspect, the invention includes biological replicas (i.e., copies of isolated DNA sequences made in vivo or in vitro) of DNA sequences of the invention. Autonomously replicating recombinant constructions such as plasmid and viral DNA vectors incorporating polynucleotides encoding chitin-binding fragments of **human chitinase,** including any of the DNAs described above, are provided. Preferred vectors include expression vectors in which the incorporated chitinase fragment-encoding cDNA is operatively linked to an endogenous or heterologous expression control sequence and a transcription terminator. Such expression vectors may further include polypeptide-encoding DNA sequences operably linked to the chitinase fragment-encoding DNA sequences, which vectors may be expressed to yield a fusion protein comprising the polypeptide of interest.

BSPR:
Knowledge of DNA sequences encoding the chitin-binding portion of **human chitinase** allows for modification of cells to permit or increase expression of the chitin-binding portions. Cells can be modified, (e.g., by homologous recombination) to provide increased expression of the chitin-binding portion of **human chitinase** by inserting all or part of a heterologous promoter in the appropriate position within the gene. The heterologous promoter is inserted in such a manner that it is operably linked to the DNA sequence encoding the chitin-binding portion of **human chitinase**. See, for example, PCT International Publication Nos. WO 94/12650, WO 92/20808 and WO 91/09955. Amplifiable marker DNA and/or intron DNA may be inserted along with the heterologous promoter DNA.

BSPR:
The invention further comprehends use of chitinase fragment products in screening for proteins or other molecules (e.g., small molecules) that specifically bind to the chitin-binding domain of **human chitinase or that modulate binding of human chitinase to chitin or to human** extracellular matrix proteins such as hyaluronic acid. Proteins or other molecules (e.g., small molecules) which specifically bind to chitinase can be identified using fragments of chitinase isolated from plasma, recombinant chitinase fragment products, or cells expressing such products. Proteins or other molecules that bind to the chitin-binding domain of chitinase may be used to modulate its activity. Binding proteins that specifically bind to the chitin-binding domain of chitinase are contemplated by the invention and include antibody substances (e.g., monoclonal and polyclonal antibodies, single chain antibodies, chimeric antibodies, humanized antibodies, human antibodies, and CDR-grafted antibodies, including compounds which include CDR sequences which specifically recognize a polypeptide of the invention). By "specifically bind to the chitin-binding domain of chitinase" it is meant that the binding protein recognizes exclusively the chitin-binding domain of chitinase and not the catalytically active portion of chitinase. Binding proteins are useful, in turn, in compositions for immunization as well as for purifying chitinase, and are useful for detection or quantification of chitinase in fluid and tissue samples by known immunological procedures. Anti-idiotypic antibodies specific for chitinase-specific antibody substances are also contemplated.

BSPR:
The scientific value of the information contributed through the disclosures of DNA and amino acid sequences of the present invention is manifest. As one series of examples, knowledge of the sequence of a cDNA for chitinase makes possible the isolation by DNA/DNA hybridization or polymerase chain reaction (PCR) of genomic DNA sequences encoding other mammalian chitinases and the like. DNA/DNA hybridization or PCR procedures carried out with DNA sequences of the invention under conditions of stringency standard in the art are likewise expected to allow the isolation of DNAs encoding **human allelic variants of chitinase, other structurally related human** proteins sharing the chitin-binding property of chitinase, and the chitin-binding regions of non-**human species proteins homologous to chitinase**. The DNA sequence information provided by the present invention also makes possible the development, by homologous recombination or "knockout" strategies [see, e.g., Kapecchi, Science, 244: 1288-1292 (1989)], of animals that fail to express a functional chitinase enzyme, overexpress chitinase enzyme, or express a variant chitinase enzyme. Such animals are useful as models for studying the in vivo activity of chitinase or modulators of chitinase. Polynucleotides of the invention when suitably labelled are useful in hybridization assays to detect the capacity of cells to synthesize chitinase. Polynucleotides of the invention may also be the basis for diagnostic methods useful for identifying a genetic alteration(s) in the chitinase locus that underlies a disease state or states. Also made available by the invention are anti-sense polynucleotides relevant to regulating expression of chitinase by those cells which ordinarily express the same.

BSPR:
The **human chitinase** fragment products of the invention are also useful as a chitin-specific reagent for specifically identifying the presence of chitin in a sample. According to this aspect of the invention, a chitinase fragment product having chitin-binding activity is conjugated with a detectable label, such as a radioisotope, fluorophore, dye, electron-dense compound, or enzyme, contacted with the sample to be tested, and analyzed qualitatively or quantitatively for the presence of chitin. "Conjugated" as used herein means linked by covalent bonds. Such techniques are well known and illustrated in, e.g., U.S. Pat. No. 5,587,292, incorporated herein by reference. The amount of chitin thus measured can be indicative of the fungal load in an infected patient. Two preferred fragments for use according to this method are the 54 amino acid chitin-binding domain consisting of amino acid residues 392 through 445 of the **human chitinase** amino acid sequence set out in SEQ ID NO: 2 and the 49 amino acid chitin-binding domain consisting of amino acid residues 397 through 445 of SEQ ID NO: 2.

BSPR:
Specifically contemplated by the invention are compositions comprising chitinase fragment products for use in methods for treating a mammal susceptible to or suffering from fungal infections. It is contemplated that the chitinase fragment products may be conjugated to other conventional anti-fungal agents, including amphotericin B and the structurally related compounds nystatin and pimaricin; 5-fluorocytosine; azole derivatives such as fluconazole, ketoconazole, clotrimazole, miconazole, econazole, butoconazole, oxiconazole, sulconazole, terconazole, itraconazole and tioconazole;

allylamines-thiocarbamates, such as tolnaftate, naftifme and terbinafme; griseofulvin; ciclopirox olamine; haloprogin; undecylenic acid; and benzoic acid. [See, e.g., Goodman & Gilman, The Pharmacological Basis of Therapeutics, 9th ed., McGraw-Hill, NY (1996).] According to this aspect of the invention, the chitin-binding fragment products serve as a vector to target known fungicidal or fungistatic compounds to pathogenic chitin-bearing fungi, and thus may improve the effectiveness of these conventional anti-fungal agents, perhaps by rendering the fungi more susceptible to their action. A reduction in the amount of conventional anti-fungal agent needed to exert the desired therapeutic effect may allow the drugs to be used at less toxic levels. The ability to selectively target fungi or yeast using a chitin-binding domain fragment also allows administration of such fragments conjugated to cytotoxic agents that are not themselves selectively anti-fungal. This aspect of the invention contemplates conjugation of chitin-binding chitinase fragments to any cytotoxic agent known in the art, including radioisotopes (such as 90Y, 188Re, 186Re, 199Au, 64Cu, 67Cu, 131I), toxins and chemotherapeutic agents, that would be effective against yeast. Suitable cytotoxic agents can be easily identified using methods known in the art. Using **human chitinase** chitin-binding domain for this purpose is more advantageous than using chitin-binding domains of **chitinases of other species because human** polypeptides are expected to be non-immunogenic in humans.

BSPR:
The **human chitinase** cDNA has been isolated from a macrophage cDNA library. Macrophages are known to be closely associated with rheumatoid arthritis lesions [Feldman et al., Cell, 85:307-310 (1996)], and macrophage products such as TNP-.alpha. are implicated in disease progression. A protein with homology to **human chitinase,** C-gp39, has been detected in the synovium and cartilage of rheumatoid arthritis patients. While the natural substrate for **human chitinase** is probably chitin from pathogenic organisms, the enzyme may also exhibit activity on endogenous macromolecules which form the natural extracellular matrix. For example, it has been suggested that hyaluronic acid, a major component of the extracellular matrix, contains a core of chitin oligomers. [Semino et al., Proc. Nat'l Acad. Sci., 93:4548-4553 (1996); Varki, Proc. Nat'l. Acad. Sci., 93:4523-4525 (1996).] Chitinase may therefore be involved in degradation of extracellular matrix in diseases such as rheumatoid arthritis. The role of chitinase may be determined by measuring chitinase levels and/or the effects of chitinase administration or chitinase inhibition in synovial fluid isolated from arthritic joints. Endogenous chitinase levels can be measured by enzymatic assay or with an antibody. Viscosity of synovial fluid can be measured before and after chitinase treatment; a decrease of viscosity associated with chitinase would be consistent with an endogenous chitinase substrate. Modulation of chitinase activity could thereby modulate the progression of joint destruction in rheumatoid arthritis.

DEPR:
Other aspects and advantages of the present invention will be understood upon consideration of the following illustrative examples. Example 1 describes the isolation of **human chitinase cDNA clones from a human** macrophage cDNA library. Example 2 addresses the pattern of **chitinase gene expression in various human** tissues. Example 3 describes the recombinant expression of the **human chitinase** gene in prokaryotic cells and purification of the resulting enzyme. Example 4

provides a protocol for the recombinant production of **human chitinase** in yeast. Example 5 describes the recombinant expression of the **human chitinase** gene in mammalian cells and purification of the resulting protein. Example 6 describes production of **human chitinase** polypeptide analogs and fragments by peptide synthesis or recombinant production methods. Example 7 describes production of **human chitinase** fragments having chitin-binding activity and analogs thereof. Example 8 provides a protocol for generating monoclonal antibodies that are specifically immunoreactive with **human chitinase**. Example 9 describes an assay for the measurement of chitinase catalytic activity. Example 10 addresses determination of the anti-fungal activity of test drugs in vitro. Example 11 addresses determination of the anti-fungal activity of test drugs in vivo in a mouse model, and Examples 12 through 15 address rabbit models of invasive aspergirosis, disseminated candidiasis, Candida ophthahmitis, and Candida endocarditis. Example 16 compares chitin-binding and chitin hydrolysis activities of full length **human chitinase** and a C-terminally truncated fragment. Example 17 addresses conjugation of chitin-binding fragments to other moieties.

DEPR:
The nucleotide and deduced amino acid sequence of these cDNA clones were compared to sequences in nucleotide and peptide sequence databases to determine similarity to known genes. Sequence comparisons were performed by the BLAST Network Service of the National Center for Biotechnology Information using the alignment algorithm of Altschul et al., J. Mol. Biol., 215:403-410 (1990). Clone MO-911 exhibited significant homology to several different sequences, including mouse macrophage secretory protein YM-1 precursor (Genbank accession no. M94584), human cartilage gp-39 (Hakala et al., supra), oviductal glycoprotein from sheep, cow, and humans (DeSouza et al., supra), and chitinases from parasite (Oncocerca, Genbank accession no. U14639), wasp (Chelonus, Genbank accession no. U10422), plant (Nicotiana, Genbank accession no. X77111), and bacteria (Serratia, Genbank accession no. Z36295); its highest observed homology was to mammalian genes that encoded proteins with chitinase homology but no demonstrated chitinase activity. Further sequence analysis of MO-911 suggested that it contained a portion of the coding region for a **human chitinase** homolog.

DEPR:
The DNA sequence of clone pMO-218 (deposited on Jun. 7, 1996 under the terms of the Budapest Treaty with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md. 20852, U.S.A. under Accession No. 98077) is set forth in SEQ ID NO: 1, and the encoded amino acid sequence is set forth in SEQ ID NO: 2. MO-218 appeared to include the entire coding region of the **human chitinase** cDNA (nucleotides 2 to 1399 of SEQ ID NO: 1), which comprises a twenty-one amino acid putative signal sequence followed by 445 encoded amino acids (residues 1 to 445 of SEQ ID NO: 2). The twenty-two amino acids following the putative signal sequence exactly match the amino-terminal sequence of purified **human chitotriosidase** reported in Renkema et al., supra. Renkema et al. also described a twenty-one amino acid sequence from a tryptic fragment of **human chitotriosidase** which corresponds exactly to residues 157 to 177 of MO-218 (SEQ ID NO: 2). Boot et al., supra, report the cloning of a **human chitotriosidase** cDNA which contains a coding sequence essentially identical to that of MO-218. The sequence of MO-218 differs from Boot et al.

by an additional fourteen nucleotides at the 5' end and by a nucleotide change at nucleotide 330 in the coding region.

DEPR:
Northern blot analysis was performed to identify tissues in which the **human chitinase** is expressed. A multiple human tissue Northern blot (Clontech, Palo Alto, Calif.) was hybridized with the entire coding region of MO-218 under standard stringent conditions (according to the Clontech laboratory manual). Greatest hybridization was observed to lung tissue (+++) and ovary (+++), with much smaller levels (+) in thymus and placenta. The size of the hybridizing mRNA was 2.0 kb for lung, ovary and thymus, which corresponds well with the size of the cloned cDNA (1.6 kb, or about 1.8 kb including the polyA tail). The size of the hybridizing placental mRNA was considerably smaller, at 1.3 kb. Chitinase hybridization was not observed in spleen, prostate, testes, small intestine, colon, peripheral blood leukocytes, heart, brain, liver, skeletal muscle, kidney, or pancreas. Chitinase expression in lung is consistent with a protective role against pathogenic organisms that contain chitin, since the lung represents the primary route of entry for fungal pathogens.

DEPR:
Transformants containing the resulting expression plasmid (pAraMO218) were induced with arabinose and grown at 37.degree. C. These transformants produced inclusion bodies containing a 39 kDa protein which was a truncated form of chitinase (engineered to contain 373 instead of 445 amino acids). This chitinase fragment contains four cysteine residues, while the full length chitinase contains ten cysteine residues. The inclusion bodies were separated from the E. coli culture and electrophoresed on SDS-PAGE. The 39 kDa band was transferred to a PVDF membrane and amino terminal sequenced. The majority (about two-thirds) of the material contained a sequence corresponding to the amino terminus of **human chitinase**. The remaining material corresponded to a contaminating E. coli protein, porin. This recombinant chitinase preparation from E. coli was useful for producing polyclonal and monoclonal antibodies (described below in Example 8).

DEPR:
Exemplary protocols for the recombinant expression of **human chitinase** in yeast and for the purification of the resulting recombinant protein follow. The coding region of **human chitinase** is engineered into vectors for expression in Saccharomyces cerevisiae using either PCR or linker oligonucleotides designed to encode a fusion polypeptide containing a secretion mediating leader to the coding region for **human chitinase** corresponding to the amino terminus of the natural molecule. Secretion signal peptides include, e.g., SUC2 or equivalent leaders with a functional signal peptidase cleavage site, or pre-pro-alpha factor or other complex leader composed of a pre, or signal peptide, and a pro, or spacer region, exhibiting a KEX2 cleavage site. The DNA encoding the signal sequence can be obtained by oligonucleotide synthesis or by PCR. The DNA encoding the pre-pro-alpha factor leader is obtained by PCR using primers containing nucleotides 1 through 20 of the alpha mating factor gene and a primer complementary to nucleotides 255 through 235 of this gene [Kujan and Herskowitz, Cell, 30:933-943 (1982)]. The pre-pro-alpha leader coding sequence and **human chitinase** coding sequence fragments are ligated into a plasmid containing the yeast alcohol dehydrogenase (ADH2) promoter, such that the promoter directs the expression of a fusion protein. As taught by Rose and

Broach, [Meth. Enz., 185:234-279, D. Goeddel, ed., Academic Press, Inc., San Diego, Calif. (1990)], the vector further includes an ADH2 transcription terminator downstream of the cloning site, the yeast "2-micron" replication origin, a selectable marker, for example TRP1, CUP1 or LEU2 (or LEU2-d) or other equivalent gene, the yeast REP1 and REP2 genes, the E. coli beta lactamase gene, and an E. coli origin of replication. The beta-lactamase and TRP1 genes provide for selection in bacteria and yeast, respectively. The REP1 and REP2 genes encode proteins involved in plasmid copy number replication.

DEPR:
The DNA constructs described in the preceding paragraphs are transformed into yeast cells using a known method, e.g. lithium acetate treatment [Stearns et al., Meth. Enz., supra, pp. 280-297] or by equivalent methods. The ADH2 promoter is induced upon exhaustion of glucose in the growth media [Price et al., Gene, 55:287 (1987)]. The pre-pro-alpha sequence or other leader sequence effects secretion of the fusion protein, releasing the mature **human chitinase** peptide from the cells. The signal peptide leader is processed by signal peptidase or, in the case of pre-pro-alpha removal of the pro region, by the KEX2 protease [Bitter et al., Proc. Natl. Acad. Sci. USA, 81:5330-5334 (1984)].

DEPR:
The secreted recombinant **human chitinase** is purified from the yeast growth medium by, e.g., the methods used to purify chitinase from bacterial and mammalian cell supernatants (see Example 3 above and Example 5 below).

DEPR:
The MO-218 clone and the MO-13B clone, both of which contain full length **human chitinase** cDNA 3' to the CMV promoter of pRc/CMV, were isolated. A third plasmid, which corresponded to the same C-terminal fragment expressed in bacterial cells in Example 3 above, was prepared as follows. The MO-218 plasmid was amplified by PCR using oligonucleotide primer 218-1 (CGCAAGCTTGAGAGCTCCGTrCCGCCACATGGTGCGGTCTGTGGCCTGGG, SEQ ID NO: 12), which
contains a Hind III site and nucleotides 2 through 23 of the MO-218 chitinase cDNA of SEQ ID NO: 1, and with complementary downstream primer T-END (GACTCTAGACTAGGTGCCTGAAGGCAAGTATG, SEQ ID NO: 13), which contains nucleotides
1164 through 1183 of MO-218, a stop codon and an Xbal site. The amplified DNA was purified by electrophoresis, digested with Xbal and HindIII, and cloned into the pRc/CMV vector (Invitrogen, San Diego, Calif.) previously cut with the same restriction enzymes. The junctions of the resulting clone was sequenced on a Model 373 (Applied Biosystems, Foster City, Calif.), confirming that the clone encoded the predicted engineered protein sequence, set forth in SEQ ID NO: 14.

DEPR:
Recombinant **human chitinase** was purified as follows. Five days after transfection of COS cells with the pRc/CMV-MO-13B plasmid, conditioned media from the culture was harvested and diluted with an equal volume of water. The diluted conditioned media was applied to a Q-Sepharose Fast Flow column (Pharmacia Biotech, Uppsala, Sweden) which was pre-equilibrated in 25 mM Tris, 10 mM sodium chloride, 1 mM EDTA, at pH 8.0. Approximately 95% of the

chitinase activity flowed through and did not bind to the column. This Q-Sepharose flow through was adjusted to 1.2 M ammonium sulfate and applied to a Butyl-Sepharose 4 Fast Flow column (Pharmacia) which was pre-equilibrated in 25 mM Tris, 1.2 M ammonium sulfate, 1 mM EDTA, at pH 8.0. Protein was eluted using a reverse gradient of 1.2 M to 0 M ammonium sulfate in 25 mM Tris, pH 8.0. A single absorbance peak at 280 nm corresponding to the chitinase activity peak was eluted at low salt. This material was greater than 85% pure as determined by SDS-PAGE and contained approximately 60% of the chitinase activity. The protein was then concentrated and buffer exchanged into 20 mM Tris, 150 mM sodium chloride, at pH 8.0 using a 10,000 MWCO membrane (Ultrafree.TM. 10K, Millipore Corp., Bedford, Mass.). This preparation was then tested for enzymatic and anti-fungal activity in vitro as described in Examples 9 and 10 below. The recombinant preparation had a chitotriosidase activity of 90 nmol/min per mg protein.

DEPR:
The supernatant from the pHDEF1/CTN.1 transfected CHO cells containing overexpressed recombinant **human chitinase** (rH-Chitinase) was purified as follows. In preparation for anion exchange chromatography, the supernatant was diluted 1:3 with 20 mM Tris, pH 7.0 (Buffer A). An anion exchange column, packed with Q-Sepharose Fast Flow Resin (Pharmacia Biotech Inc., Piscataway, N.J.), was equilibrated with Buffer A and loaded with 15L diluted supernatant per 1L resin. The rH-Chitinase was collected in the Flow Through from the Q-Sepharose column and adjusted to 5% Polyethylene Glycol (PEG) 400 (Malinckrodt Baker, Inc., Phillipsburg, N.J.), 30 mM sodium acetate, pH 4.3 in preparation for cation exchange chromatography. A cation exchange column, packed with CM-Sepharose Fast Flow Resin (Pharmacia Biotech Inc., Piscataway, N.J.), was equilibrated with 30 mM sodium acetate, 5% PEG 400, pH 4.3 (Buffer B). The rH-Chitinase sample was loaded onto the CM-Sepharose column at 1 mg per mL resin, and rH-Chitinase was eluted from the column with 40 mM Tris, 5% PEG 400, pH 7.5 (Buffer C). The rH-Chitinase sample was then prepared for hydrophobic interaction chromatography by adding (NH.sub.4).sub.2 SO.sub.4 to 1.5 M. A column packed with Macro-Prep Methyl H1C Support, (Bio-Rad Laboratories, Hercules, Calif.,) was equilibrated with 20 mM Tris, 5% PEG 400, pH 7.0 (Buffer D) containing 1.5 M (NH.sub.4).sub.2 SO.sub.4. The rH-Chitinase sample was loaded onto the Macro-Prep Methyl column at 1 mg per mL resin. The column was washed with Buffer D containing 1.1 M (NH.sub.4).sub.2 SO.sub.4, and rH-Chitinase was eluted with Buffer D containing 0.2 M (NH.sub.4).sub.2 SO.sub.4. The purified eluate was exchanged into 150 mM NaCl, 20 mM Tris, pH 7.0 (Buffer E) by membrane filtration.

DEPR:
Recombinant techniques such as those described in the preceding examples may be used to prepare **human chitinase** polypeptide analogs or fragments. More particularly, polynucleotides encoding **human chitinase** are modified to encode polypeptide analogs of interest using well-known techniques, e.g., site-directed mutagenesis and polymerase chain reaction. C-terminal and N-terminal deletions are also prepared by, e.g., deleting the appropriate portion of the polynucleotide coding sequence. See generally Sambrook et al., supra, Chapter 15. The modified polynucleotides are expressed recombinantly, and the recombinant polypeptide analogs or fragments are purified as described in the preceding examples.

DEPR:
Residues critical for **human chitinase** activity are identified, e.g., by homology to other **chitinases and by substituting alanines for the native human chitinase** amino acid residues. Cysteines are often critical for the functional integrity of proteins because of their capacity to form disulfide bonds and restrict secondary structure. To determine whether any of the cysteines in **human chitinase** are critical for enzymatic activity, each cysteine is mutated individually to a serine.

DEPR:
A 39 kDa C-terminally truncated fragment of the mature **human chitinase** protein was prepared as described above in Examples 3 and 5 by introduction of a stop codon after the codon for amino acid 373. This 39 kDa fragment lacked seventy-two C-terminal amino acid residues of the mature protein, including six cysteines, yet retained similar specific enzymatic activity compared to the full length recombinant **human chitinase**. This result indicates that the missing seventy-two C-terminal residues, including the six cysteines, are not required for enzymatic activity.

DEPR:
Further C-terminal deletions may be prepared, e.g., by digesting the 3' end of the truncated **human chitinase** coding sequence described in Example 3 with exonuclease III for various amounts of time and then ligating the shortened coding sequence to plasmid DNA encoding stop codons in all three reading frames. N-terminal deletions are prepared in a similar manner by digesting the 5' end of the coding sequence and then ligating the digested fragments into a plasmid containing a promoter sequence and an initiating methionine immediately upstream of the promoter site. These N-terminal deletion analogs or fragments may also be expressed as fusion proteins.

DEPR:
Alternatively, **human chitinase** polypeptide analogs may also be prepared by full or partial chemical peptide synthesis using techniques known in the art. [See, e.g., synthesis of IL-8 in Clark-Lewis et al., J. Biol Chem., 266:23128-34 (1991); synthesis of IL-3 in Clarke-Lewis et al., Science, 231:134-139 (1986); and synthesis by ligation in Dawson et al., Science, 266:776-779 (1994).] Such synthetic methods also allow the selective introduction of novel, unnatural amino acids and other chemical modifications.

DEPR:
The biological activities, including enzymatic, anti-fungal, and extracellular matrix remodeling activities, of the **human chitinase** polypeptide analogs are assayed by art-recognized techniques, such as those described in Examples 9 to 15 below.

DEPR:
The location of the chitin-binding domain of **human chitinase** was determined by generating fusion proteins comprising N-terminally truncated portions of **human chitinase** and testing these products for chitin-binding activity. First, a chimeric protein comprising full length secreted alkaline phosphatase (SEAP) protein (at the N-terminus of the chimeric protein) [Berger et al., Gene, 66:1-10 (1988)] fused to the C-terminal 99 amino acids of **human chitinase** (at

the C-terminus of the chimeric protein) was generated as follows. The SEAP component acts as a traceable marker of the chimeric protein.

DEPR:
The SEAP DNA was amplified from the pSEAP2-Control plasmid (Clontech, Palo Alto, Calif.) via polymerase chain reaction (PCR) with primers SEAP Start (SEQ ID NO: 18) and SEAP Stop (SEQ ID NO: 19) that introduced a HindIII site to the 5' end and a multiple cloning region to the 3' end. PCR was carried out using 100 ng of template DNA, 1 .mu.g of each primer, 0.125 mM of each dNTP, 10 mM Tris-HCl, pH 8.4, 50 mM MgCl.sub.2 and 2.5 units of Taq polymerase, with an initial denaturation step of 94.degree. C. for four minutes followed by 30 cycles of amplication: 1 minute at 94.degree. C., 1 minute at 60.degree. C., and 2 minutes at 72.degree. C. This PCR-generated cDNA was cloned into the HindIII and ApaI sites of pcDNA3 (Invitrogen, San Diego, Calif.) to generate a vector called pcDNA-SEAP. DNA encoding the C-terminal 99 amino acids of **human chitinase** (residues 347-445) was also generated by PCR under the same conditions using the primers indicated in Table 1 below, which introduced EcoRI and XbaI sites to the 5' and 3' ends. This PCR-generated chitinase DNA sequence was cloned into the EcoRI and AbaI sites of the multiple cloning region of pcDNA-SEAP.

DEPR:
The resulting construct encoding the chimera was transiently transfected into COS 7 cells by incubation in Dulbecco's modified Eagle medium (DMEM) containing 0.5 mg/ml DEAE dextran, 0.1 mM chloroquine and 10 .mu.g of plasmid DNA for 1.5 hours. The cells then were treated with 10% DMSO in phosphate buffered saline for 45 seconds, washed with serum-free medium and incubated in DMEM supplemented with 1 mM L-glutamine, 100 U/ml penicillin, 100 .mu.g/ml streptomycin and 10% fetal calf serum. After four days, the culture medium was assayed for SEAP activity as described by Flanagan and Leder, Cell, 63:185-194 (1990). SEAP activity was readily detectable. Incubation of the culture medium containing this fusion protein with insoluble chitin (Sigma, St. Louis, Mo.) for 1 hour at 4.degree. C. resulted in precipitation of more than 80% of the SEAP activity with the chitin. This result demonstrated that the entire chitin-binding domain is contained within the C-terminal 99 amino acids of **human chitinase**.

DEPR:
To determine whether any of the six cysteines within the 99 C-terminal amino acids of **human chitinase** were critical for binding chitin, analogs of chitinase fragments were prepared in which each cysteine was mutated individually to a serine. Six PCR products in which each of the six cysteines was individually mutated to serine were generated using the primers indicated in Table 2 below and fused to SEAP cDNA as described above. Chimeric proteins produced by transiently transfected COS cells were assayed for chitin-binding activity as described above. The results of these experiments demonstrated that each of the six cysteines is required for chitin-binding activity.

DEPR:
A chitin-binding domain fragment consisting of residues 392-445 of SEQ ID NO: 2 was expressed at high levels in the yeast Saccharomyces cerevisiae. An expression construct, .alpha.-FLAG-CBD, was designed in which the nucleotides corresponding residues 392-445 of SEQ ID NO: 2 were fused to the 3' terminus of

sequence encoding the S. cerevisiae .alpha.-factor pre-pro sequence [Brake et al., Proc. Natl. Acad. Sci. 81:4642-4646 (1984)] and the FLAG epitope tag (Eastman Kodak). To accomplish this, PCR using primers CBD.alpha.FLAG (sense; SEQ ID NO: 33) and Hu Chit Stop 5 (antisense; SEQ ID NO: 34) was conducted using full-length **human chitinase** DNA as a template. The CBD.alpha.FLAG primer sequence contains an Asp 718 restriction endonuclease site upstream of a FLAG tag-encoding region that is in-frame with the sequence that encodes the first eight amino acids of the chitin-binding domain fragment 392-445. The Hu Chit Stop 5 primer sequence encodes the C-terminal seven amino acids of the chitin-binding domain fragment followed by Gly-Ala-Gly linked to six histidine residues (His.sub.6) which precede a three amino acid segment prior to the translation termination codon. The His.sub.6 tract is included to facilitate purification of the expressed product by metal affinity chromatography [as described in Nilsson et al., Prot. Expr. Punification 11:1-16 (1997)]. A Not I restriction endonuclease site was included immediately 3' of the stop codon.

DEPR:
The following two protocols (multiple challenge or single shot immunizations) may be used to generate monoclonal antibodies to **human chitinase**. In the first protocol, a mouse is immunized by periodic injection with recombinant **human chitinase** (e.g., 10-20 .mu.g emulsified in Freund's Complete Adjuvant) obtained as described in any of Examples 3 through 6. The mouse is given a fmal pre-fusion boost of **human chitinase** in PBS, and four days later the mouse is sacrificed and its spleen removed. The spleen is placed in 10 ml serum-free RPMI 1640, and a single cell suspension is formed by grinding the spleen between the frosted ends of two glass microscope slides submerged in serum-free RPMI 1640, supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin, and 100 .mu.g/ml streptomycin (RPMI) (Gibco, Canada). The cell suspension is filtered through sterile 70-mesh Nitex cell strainer (Becton Dickinson, Parsippany, N.J.), and is washed twice by centrifuging at 200 g for 5 minutes and resuspending the pellet in 20 ml serum-free RPMI. Splenocytes taken from three naive Balb/c mice are prepared in a similar manner and used as a control. NS-1 myeloma cells, kept in log phase in RPMI with 11% fetal bovine serum (FBS) (Hyclone Laboratories, Inc., Logan, Utah) for three days prior to fusion, are centrifuged at 200 g for 5 minutes, and the pellet is washed twice as described in the foregoing paragraph.

DEPR:
On days 2, 4, and 6, after the fusion, 100 .mu.l of medium is removed from the wells of the fusion plates and replaced with fresh medium. On day 8, the fusion is screened by ELISA, testing for the presence of mouse IgG binding to **human chitinase** as follows. Immulon 4 plates (Dynatech, Cambridge, Mass.) are coated for 2 hours at 37.degree. C. with 100 ng/well of **human chitinase** diluted in 25 mM Tris, pH 7.5. The coating solution is aspirated and 200 ul/well of blocking solution [0.5% fish skin gelatin (Sigma) diluted in CMF-PBS] is added and incubated for 30 min. at 37.degree. C. Plates are washed three times with PBS with 0.05% Tween 20 (PBST) and 50 .mu.l culture supernatant is added. After incubation at 37.degree. C. for 30 minutes, and washing as above, 50 .mu.l of horseradish peroxidase conjugated goat anti-mouse IgG(fc) (Jackson ImmunoResearch, West Grove, Pa.) diluted 1:3500 in PBST is added. Plates are incubated as above, washed four times with PBST, and 100 .mu.L substrate, consisting of 1 mg/ml o-phenylene diamine (Sigma) and 0.1 .mu.l/ml 30% H.sub.2 O.sub.2 in 100 mM citrate, pH 4.5, are added. The color

reaction is stopped after 5 minutes with the addition of 50 .mu.l of 15% H.sub.2 SO.sub.4. A.sub.490 is read on a plate reader (Dynatech). Selected fusion wells are cloned twice by dilution into 96-well plates and visual scoring of the number of colonies/well after 5 days. The monoclonal antibodies produced by hybridomas are isotyped using the Isostrip system (Boehringer Mannheim, Indianapolis, Ind.).

DEPR:
Alternatively, a second protocol utilizing a single-shot intrasplenic immunization may be conducted generally according to Spitz, Methods Enzymol., 121:33-41 (1986). The spleen of the animal is exposed and recombinant **human chitinase** (e.g., 10-20 .mu.g in PBS at a concentration of about 0.02% to 0.04%, with or without an aluminum adjuvant), obtained as described in any of Examples 3 through 6, is injected, after which the spleen is returned to the peritoneal cavity and the animal is stitched closed. Three days later, the mouse is sacrificed and its spleen removed. A spleen cell suspension is prepared, washed twice with RPMI 1640 supplemented with 3% fetal calf serum (FCS), and resuspended in 25 ml of the same medium. Myeloma cells (NS-O) are collected at logarithmic growth phase, washed once and added to the spleen cell suspension in a 50 ml tube, at a ratio of 3:1 or 2:1 (spleen cells:myeloma cells). The mixture is pelleted at about 450.times.g (1500 rpm), the supernatant aspirated, and the pellet loosened by tapping the tube. Fusion is performed at room temperature by adding 1 ml of polyethylene glycol (PEG) 1500 over 1 minute, with constant stirring. The mixture is incubated for another minute, then 1 ml of warm RPMI (30 to 37.degree. C.) is added over 1 minute followed by 5 ml RPMI over 3 minutes and another 10 ml RPMI over another 3 minutes. The cell suspension is centrifuged and resuspended in about 200 ml of HAT selective medium consisting of RPMI 1640 supplemented with 100 U/ml penicillin, 100 .mu.g/ml streptomycin, 20% FCS, 100 .mu.M hypoxanthine, 0.4 .mu.M aminopterin and 16 .mu.M thymidine. The cell suspension is dispensed in 1 ml volumes into tissue culture plates and incubated at 37.degree. C. in a humid atmosphere with 5% CO.sub.2 -95% air for 8 to 10 days. Supernatants are aspirated and the cells are fed with 1 ml HAT medium per well, every 2 to 3 days according to cell growth. Supernatants of confluent wells are screened for specific antibodies and positive wells are cloned.

DEPR:
Using the above protocols, several monoclonal antibodies with reactivity to **human chitinase** were generated. For fusion 243, each of five 6-12 week old Balb/c mice was prebled on day 0 and then immunized by subcutaneous injection with 10-20 .mu.g recombinant **human chitinase** prepared as described in Example 5, emulsified in complete Freunds adjuvant. On days 21, 42 and 60 each mouse was boosted with 50 .mu.g of the same recombinant **human chitinase** in incomplete Freunds adjuvant. Mouse #2483 was additionally given 20 .mu.g of recombinant **human chitinase** daily on days 216 through 219. On day 220 the spleen of mouse #2483 was removed sterilely and treated as described above. Briefly, a single-cell suspension was formed by grinding the spleen between the frosted ends of two glass microscope slides submerged in serum free RPMI 1640, supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin, and 100 .mu.g/ml streptomycin (RPMI) (Gibco, Canada). The cell suspension was filtered through a sterile cell strainer (Becton Dickinson, Parsippany, N.J.), and washed twice with serum free RPMI by centrifuging at 200.times.g for 5 minutes and resuspending the pellet in 20 ml serum free RPMI.

Thymocytes taken from naive Balb/c mice were prepared in a similar manner.

DEPR:
Supernatants from fusion 243 were screened initially by ELISA on the immunogen (full length **human chitinase**), and detected with goat anti-mouse IgG (fc) horseradish peroxidase conjugate. To ensure clonality, positive wells chosen from each fusion were subdloned 4 times by limiting dilution, using media lacking aminopterin. Cloning was completed for cell lines 243K, 243M and 243Q.

DEPR:
The **chitotriosidase activity of the recombinant human chitinase** produced in COS cells as described in Example 5A was determined to be 90 nmol/min per mg protein. Any of the **human chitinase** fragment products of the present invention can also be tested for chitinase enzymatic activity in this manner.

DEPR:
Conventional anti-fungal agents that have been conjugated to **human chitinase** products of the invention can be tested for inhibition of fungal growth in vitro. The two fungi Candida albicans and Aspergillus fumigatis are serious pathogens for immunocompromised patients. Both Candida and Aspergillus are grown in RPMI growth media at 37.degree. C. to approximately 10,000-50,000 colony forming units (CFU) per ml. Serial dilutions of the test drug are added to cultures, and fungal growth is assessed at 24 hours by turbidity of cultures.

DEPR:
The pharmacokinetics of recombinant **human chitinase** in mice were determined as follows. Female Balb/c mice, 6-8 weeks old, were administered 0.5 mg/kg, 5.0 mg/kg and 50 mg/kg recombinant **human chitinase** by intravenous injection in the tail vein. For each dose, mice were terminally bled at 0.01, 0.25, 1, 8 and 24 hours after injection (2 animals were used per time point per dosage). Serum samples were then assayed for chitinase activity and concentration. Results are shown in Table 3 below.

DEPR:
These observations indicate that the C-terminal 72 amino acids of **human chitinase** are required for chitin-binding activity but not for hydrolysis of triacetylchitotriose.

DEPR:
These observations demonstrate that the C-terminal 72 amino acids of **human chitinase** are required for hydrolysis of chitin.

DEPR:
In order to test whether the chitin-binding domain is amenable to serving as a carrier for small molecule pharmaceuticals, a chitin-binding domain consisting of amino acids 392-445 of **human chitinase** was chemically conjugated with either biotin or rhodamine, as follows.

DEPC:
Chitinase Gene Expression Pattern in Human Tissues

DEPC:
Production of Recombinant **Human Chitinase** in Bacterial Cells

DEPC:
Production of Recombinant **Human Chitinase** in Yeast Cells

DEPC:
Production of Recombinant **Human Chitinase** in Mammalian Cells

DEPC:
Production of **Human Chitinase** Analogs and Fragments

DEPC:
Production of **Human Chitinase** Chitin-binding Fragments and Analogs Thereof

DEPC:
Preparation of Monoclonal Antibodies to **Human Chitinase**

DEPC:
Chitin-binding and Chitin Hydrolytic Activity of Fragments of **Human Chitinase**

ORPL:
Argueso et al., "Effect of the Enzymes **Chitinase and Neuraminidase on the Structure of Human** Ocular Mucus," Investigative Ophthalmology & Visual Science, 36(4):S997 (Mar. 15, 1995) (Abstract 4615-596).

ORPL:
Boot et al., "Cloning of cDNA Encoding **Chitotriosidase, Human Chitinase** Produced by Macrophage," J. Biol. Chem., 270(44):26252-26256 (Nov. 3, 1995).

ORPL:
Escott et al., "**Chitinase Activity in Human** Serum and Leukocytes," Infect. Immun., 63(12):4770-4773 (Dec., 1995).

ORPL:
Overdijk et al., "**Human Serum Contains a Chitinase:** Identification of an Enzyme, Formerly Described as 4-Methylumbelliferyl-tetra-N-Acetylchitotetraoside Hydrolase (MU-TACT Hydrolase)," Glycobiology, 4(6):797-803 (1994).

ORPL:
Renkema et al., "Synthesis, sorting, and processing into distinct isoforms of **human macrophage chitotriosidase,**" Eur. J. Biochem., 244(2):279-285 (1997).

ORPL:
Renkema et al., "Purification and Characterization of **Human Chitotriosidase,** a Novel Member of the Chitinase Family of Proteins," J. Biol. Chem., 270(5):2198-2202 (Feb. 3, 1995).

ORPL:
Renkema et al., "**Chitotriosidase, a chitinase, and the 39-kDa human** cartilage glycoprotein, a chitin-binding lectin, are homologues of family 18 glycosyl

hydrolases secreted by human macrophages," Eur. J. Biochem., 251:504-509 (Jan., 1998).

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TITLE: Method and system for providing real-time, in situ biomanufacturing process monitoring and control in response to IR spectroscopy
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INVENTOR-INFORMATION:

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ABSTRACT:

A method and system for providing real-time, biomanufacturing process monitoring and control in response to infra-red (IR) spectroscopic fingerprinting of a biomolecule. IR spectroscopy is used to fingerprint an active biomolecule in situ in a biomanufacturing process. In one embodiment, Fourier Transform Infra-red spectroscopy (FTIR) is used to determine whether an active or aged biomolecule is present in stages of a biomanufacturing process. In one preferred example, the biomanufacturing process manufactures a biomaterial in bulk. The biomanufacturing process has four stages: bioproduction, recovery, purification, and bulk storage. FTIR spectroscopy is used to monitor the optimization of each process step by providing feedback controls, and to fingerprint in real-time, in situ whether active biomolecules are present in each stage.

27 Claims, 13 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 13

DATE FILED: July 14, 2000

---------- KWIC ---------

DETL:

TABLE 1 Biomolecule Reference Interleukin-1 beta converting enzyme-like apoptotic EP-807686-A2 protease-7 Human ICE LAP-6 polypeptide EP-808904-A2 Human cytokine/peptide receptor, HR-1 receptor EP-812913-A2 Human cathepsin K gene EP-812916-A2 Homo sapiens arginase II gene EP-825260-A2 Human integrin ligand polypeptide ITGL-TSP EP-874050-A2 HPMBQ91 EP-892053-A2 cDNA encoding human MYH J10057076-A Death domain containing receptor polypeptide J11000170-A (DR3-V1) cDNA encoding human homologue of E. coli AlkB US5618717-A gene, hABH cDNA encoding human oxalyl-CoA decarboxylase US5635616-A cDNA encoding human ubiquitin conjugating enzyme US5650313-A 7 Human GABA-A receptor epsilon subunit US5654172-A Human MutT2 (hMutT2) protein US5695980-A cDNA sequence of the putative mature potassium US5710019-A channel 1 protein Human elastase IV US5710035 Nucleotide sequence of human inositol mono- US5716806-A phosphatase-H1 Human lymphoid-specific transcription factor NERF-1 US5721113-A Human DNA topoisomerase I alpha US5723311-A Human FGF-13 US5728546-A Polynucleotide sequence of a colon-specific gene US5733748-A Human vascular

IBP-like growth factor US5747280 Human AlkB polypeptide US5747312 Nucleotide sequence of the G-protein coupled receptor US5750370-A G-protein coupled prostate tissue receptor designated US5756309-A HPRAJ70 Neurotransmitter transporter US5759854 Human fibroblast growth factor 11 US5763214-A Human G-protein coupled receptor US5763218-A Nucleotide sequence of fibroblast growth factor-15 US5773252-A (FGF-15) Nucleotide sequence of the human G-protein US5776729-A chemokine receptor HGBER32 Human small CCN-like growth factor US5780263-A Arginase II US5780286 Human geranyl geranyl pyrophosphate synthase US5786193-A Human prostatic specific reductase US5786204 Paraoxonase polypeptides and use therefor US5792639 Amine transporter US5798223-A Human G-protein adrenergic receptor US5817477-A Nucleic acids and cells for recombinant production of US5817485 fibroblast growth factor-10 Human DNase US5830744-A Corpuscles of stannius protein stanniocalcin US5837498 Human cytostatin I polypeptide US5844081-A Ubiquitin conjugating enzyme (UCE) 7 US5849286-A Human elastate IV US5851814 Human DNA ligase III US5858705-A Human amine transporter US5859200 Human C5a receptor protein US5861272-A Cathepsin K gene US5861298 cDNA sequence of a human colon specific gene US5861494-A Polynucleotide encoding a human chemotactic protein US5866373 DNA encoding retinotic acid receptor epsilon US5869284 Human G-protein coupled receptor, HCEGH45 US5869632-A Superoxide dismutase-4 US5871729 Human NAF-1 DNA US5871969-A Human h4-IBBSV receptor DNA US5874240-A Antibodies to corpuscles of stannius protein US5877290 stanniocalcin Human chemotactic protein US5880263 Polynucleotides encoding chemokine alpha-2 US5910431 Arginase II polypeptide US5912159-A Method of purifying chemokines from inclusion bodies US5912327 Human deoxycytidine kinase 2 US5914258 Polynucleotides encoding extra cellular/epidermal US5916769 growth factor HCABA58X polypepides Human cystatin F US5919658 Polynucleotides encoding haemopoietic maturation US5922572 factor Human amine receptor US5928890 Human geranylgeranyl pyrophosphate synthetase US5928924 Human ABH US5929225 Vascular endothelial growth factor 2 US5932540 Polynucleotides encoding vascular endothelial growth US5935820 factor 2 Polynucleotides encoding human G-protein coupled US5942414 receptor HIBEF51 CD44-like protein and nucleic acids US5942417 Human oxalyl-coa decarboxylase US5945273 Human hematopoietic-specific protein US5945303 Cytostatin III nucleic acids encoding US5945309 Ubiquitin conjugating enzymes 7 8 and 9 US5945321 Human G-protein receptor HPRAJ70 US5948890 DNA encoding the chemotactic cytokine III US5952197 Macrophage inflammatory protein-3 WO9517092-A Haematopoietic maturation factor WO9519985-A1 hGHV-2(88) growth hormone splice variant WO9520398-A Human osteoclast-derived cathepsin-O WO9524182-A1 cDNA encoding stanniocalcin from Corpuscles of WO9524411-A1 Stannius Human fibroblast growth factor 10 WO9524414-A1 Human transforming growth factor alpha-H1 WO9524466-A1 polynucleotide DNA encoding vascular endothelial growth factor 2 WO9524473-A1 DNA encoding mature Bone Morphogenic Protein-10 WO9524474-A1 Human superoxide-dismutase-4 polynucleotide WO9527781-A1 Human DNase precursor WO9530428-A1 Human monocyte chemotactic protein-4 WO9531467-A1 Human macrophage migration inhibitory factor-3 WO9531468-A1 (MIF-3) Human DNA-topoisomerase-I alpha protein WO9531538-A1 Human neurotransmitter transporter protein WO9531539-A1 Human interleukin-6 splice variant DNA WO9532282-A1 Human FLAP II WO9535372-A1 Retinoic acid receptor epsilon WO9600242-A1 ICE-like apoptosis protease-1 WO9600297-A1 Human elastase IV gene WO9601270-A1 Human serum paraoxonase WO9601322-A1 Connective tissue growth factor-2 WO9601896-A Human K + channel 1 WO9603415-A1 Calcitonin gene-related peptide receptor WO9604928-A1 cDNA encoding a human calcitonin

receptor WO9605221-A Human adrenergic G-protein coupled receptor WO9605225-A1 G-protein coupled receptor WO9605226-A1 Human chemokine beta-4 WO9605856-A1 Chemokine beta-9 WO9606169-A1 Human GABA-A receptor epsilon subunit WO9606862-A Inositol-monophosphatase-H1 full-length gene WO9608557-A1 Transcription factor IIA small subunit WO9609311-A1 TAR-3 WO9611259-A1 Human endothelin-bombesin receptor WO9611946-A1 Hypoxanthine guanine phosphoribosyl transferase 2 WO9612501-A1 Human homologue of bacterial AlkB gene (hABH) WO9612791-A1 Human interleukin-I-converting enzyme-like apoptosis WO9613603-A1 protease-3 Tumour necrosis factor-gamma WO9614328-A1 DNA-ligase-III gene WO9614394-A1 Stanniocalcin alpha WO9615147-A1 hMutT2 WO9615222-A1 Human choline acetyltransferase WO9615806-A1 Human G-protein coupled receptor WO9616087-A1 Vascular IBP-like growth factor WO9617931-A1 Tissue inhibitor of metalloproteinase-4 WO9618725-A1 Human prostatic growth factor WO9618730-A1 Human deoxycytidine kinase 2 WO9621724-A1 Human geranylgeranyl pyrophoshate synthetase WO9621736-A1 (GGPPS) Prostate specific reductase WO9622360-A1 Ubiquitin conjugating enzyme 7 WO9623410-A1 Human chemokine alpha-1 WO9624668-A1 Human chemokine beta-11 WO9624668-A1 Keratinocyte growth factor 2 WO9625422-A1 Human G-protein coupled receptor WO9625432-A1 Human amine transporter WO9627009-A1 Human tumour necrosis factor receptor WO9628546-A1 Human B-cell translocation gene-2 polypeptide WO9629401-A1 Human G-protein coupled receptor GPR1 WO9630406-A1 Human DNA ligase III WO9630524-A1 Human tumour necrosis factor receptor WO9634095-A1 Neuropeptide receptor gene WO9634877-A1 cDNA encoding human cytokine beta-8: a chemo- WO9634891-A1 attractant for leukocytes Human inhibitor of apoptosis gene 1 WO9635703-A1 Human uridine diphosphate galactose-4-epimerase WO9635778-A1 Human transforming growth factor alpha HII WO9636709-A1 Human G protein gamma-3 subunit WO9637513-A1 Pineal gland specific gene-1 WO9639158-A1 Human cystatin E WO9639418-A1 Human colon specific gene CSG5 WO9639419-A1 Human criptin growth factor WO9639420-A1 Human vascular endothelial growth factor 3 WO9639421-A1 Natural killer cell enhancing factor C WO9639424-A1 Human bone morphogenic protein-10 WO9639431-A1 G-protein parathyroid hormone receptor HLTDG74 WO9639433-A1 Human G-protein receptor HGBER32 WO9639434-A1 Human G-protein receptor HPRAJ70 WO9639435-A1 Human G-protein coupled receptor HETGQ23 WO9639436-A1 Human G-protein chemokine receptor HDGNR10 WO9639437-A1 Human G-protein thrombin-like receptor WO9639438-A1 Human G-protein receptor HCEGH45 WO9639439-A1 Human amine receptor WO9639440-A1 G-protein coupled receptor WO9639441-A1 G-protein receptor, HTNAD29 WO9639442-A1 Human hepatoma-derived growth factor (HDGF-2) WO9639485-A1 cDNA encoding small CCN-like growth factor WO9639486-A1 cDNA encoding transforming growth factor alpha-HI WO9639497-A1 Human fibroblast growth factor 14 cDNA WO9639506-A1 (ATCC #97148) Human fibroblast growth factor 11 cDNA WO9639507-A1 (ATCC #97150) Fibroblast growth factor 13 WO9639508-A1 Human fibroblast growth factor 15 WO9639509-A1 Human vascular endothelial growth factor 2 WO9639515-A1 Human cytokine beta-13 cDNA (ATCC 97113) WO9639521-A1 Human chemokine beta-11 WO9639522-A1 Human colon specific protein WO9639541-A1 Human monocyte chemotactic protein-4 polypeptide WO9640762-A1 Human breast specific gene BSG15, clone HBNAC96 WO9702280-A1 Human cytostatin II WO9711970-A1 Human mammary transforming protein WO9717358-A1 Human stem cell antigen 2 WO9718224-A1 Human smooth muscle cell-derived migration factor WO9719704-A1 Growth factor receptor-binding protein 2 homologue WO9720573-A1 Grb2-1 Human osteo antiviral protein DNA WO9722623-A1 Human chemotactic cytokine I DNA WO9723640-A1 Human ATP receptor WO9724929-A1 Human Immune Cell Cytokine-like Hormone WO9725338-A1 (HLHDC84) DNA Human G-protein chemokine receptor HSATU68

WO9725340-Al Transforming growth factor alpha HIII polynucleotide WO9725349-A1 Human cytostatin I gene WO9727747-A1 Human neuronal attachment factor-1 DNA WO9729189-A1 Human chemokine beta4 WO9731098-A1 Human chemotactic cytokine III (CCIII) WO9732993-A1 DNA encoding a human h4-1BBSV receptor WO9733898-A1 cDNA encoding human Apoptosis inducing molecule-I WO9733899-A1 (AIM-I) Human tumour necrosis factor delta WO9733902-A1 Human mismatch repair MutY cDNA (hMYH gene) WO9733903-A1 Death domain containing receptor DR3-V1 WO9733904-A1 cDNA encoding human Arginase II WO9733985-A1 DNA encoding human Arginase II WO9733986-A1 Human chemotactic cytokinc II CCII genomic DNA WO9734013-A1 Human apoptosis inducing molecule II (AIM II) gene WO9734911-A1 Human endometrial specific steroid-binding factor I WO9734997-A1 DNA DNA encoding novel human cytokine WO9734998-A1 Human chemokine alpha-2 WO9735010-A1 Human chemokine alpha-3 WO9735027-A1 Human cytostatin III WO9735028-A1 Human growth factor HTTER36 WO9735870-A1 Epidermal differentiation factor WO9735976-A2 Human cystatin F polypeptide WO9736915-A1 **Human chitotriosidase** WO9736917-A1 Human cystatin F encoding sequence WO9737021-A1 Human natural killer cell activating factor II WO9737022-A1 (NKAF II) DNA Human extracellular/epidermal growth factor-like WO9738002-A1 protein Human haematopoietic-specific protein (HSP) DNA WO9738003-A1 Human extracellular/epidermal growth factor WO9738012-A1 HCABA58X Human brain P2X-1 receptor WO9741222-A1 Human DNA repair enzyme RAD WO9742209-A1 Human G-protein coupled receptor WO9744359-A1 Human G-protein coupled receptor WO9744360-A1 Human cathepsin K gene WO9747642-A1 Human HR-1 receptor WO9747741-A1 Homo sapiens cDNA encoding the HR-1 receptor WO9747742-A1 Human chemokine beta-15 gene WO9748807-A1 cDNA encoding a novel G-protein coupled receptor WO9803539-A1

DETL:
HNFDS78 Human CD33-like protein WO9806733-A1 DNA encoding a CD44-like protein WO9806839-A1 Mutated KGF-2 coding sequence KGF2delta33, WO9806844-A1 191K/Q DNA encoding a **human chitinase** alpha protein WO9806859-A1 DNA encoding a **human chitinase** alpha protein variant WO9806859-A1 Homo sapiens pancreas-derived plasminogen activator WO9807735-A1 inhibitor gene Human XAG growth factor huXAG-1 WO9807749-A1 Human T1 receptor-like ligand II WO9807754-A1 Human chemokine beta-16 WO9807862-A2 Human endokine-alpha WO9807880-A1 Human TI receptor-like ligand I WO9807881-A1 Nucleotide sequence of interleukin-19 WO9808870-A1 Human interleukin-1 receptor accessory molecule WO9808969-A1 Human chemokine alpha-4 encoding DNA WO9811138-A1 Human B-cell translocation gene-2 WO9812204-A1 Modified TR1 receptor WO9812344-A1 Human chemokine beta-11 (Ck beta-11) polypeptide WO9814477-A1 Human MPIF-1 genomic DNA WO9814582-A1 Galectin 8 WO9815624-A1 Brain-associated inhibitor of tissue plasminogen WO9816643-A1 activator Human TNF receptor related (TR2) gene WO9818824-A1 Homo sapiens neutrokine alpha protein gene WO9818921-A1 Human blue-light photoreceptor hCRY2 gene related WO9820042-A1 clone HFCAD18R Human mucosal adressin cell adhesion molecule-1(a) WO9820110-A1 DNA Human connective tissue growth factor-3 gene WO9821236-A1 Human calcitonin receptor cDNA clone HCEPR64 WO9821242-A1 Fibroblast growth factor-13 WO9823749-A1 Nucleotide sequence of human G-protein coupled WO9824900-A1 receptor Human chemokine beta-13 WO9824908-A1 DNA sequence encoding a human Prt1-like subunit WO9825957-A2 protein Homo sapiens CESP gene related EST clone WO9827932-A2 Human parotid secretory protein WO9828420-A1 Human oncogene induced secreted protein I WO9828421-A1 Human cell death adaptor molecule RAIDD WO9828422-A1 Human cortistatin cDNA from clone HEBCI67R WO9829438-A2 Human TRID genomic DNA WO9830693-A2 Human tumour necrosis factor receptor-6 alpha WO9830694-A2

Nucleotide sequence of the HSF cDNA clone 5 WO9831792-A1 Human extracellular matrix-1 gene WO9831798-A1 Nucleotide sequence encoding clone HMWGS46 of WO9831799-A2 Prohibitin receptor family Nucleotide sequence of the cDNA clone CAT-2 WO9831800-A2 (HT3SG28) I-FLICE-1 and I-FLICE-2 WO9831801-A1 Primer for FcR-I WO9831806-A2 Human TACE-like DNA WO9831818-A2 Human DR4 genomic DNA WO9832856-A1 Vector pHE4-5 containing human MOGp WO9833912-A1 Human breast cancer specific gene 1 (BCSG1) WO9833915-A1 Human tissue factor pathway inhibitor-3 (TFPI-3) WO9833920-A2 Dendritic cell-derived growth factor (DCDGF) WO9835039-A1 Human ELL2 cDNA EST AA545429 WO9837194-A1 Human T1-receptor ligand III clone HSRDN17R DNA WO9838311-A1 sequence Human secreted protein gene 3 clone HTGEW86 WO9839446-A2 Human secreted protein gene 100 clone HLQAB52 WO9839448-A2 Human secreted protein gene 27 clone H2MBT68 WO9840483-A2 Human death domain containing receptor 5 (DR5) WO9841629-A2 SV40 promoter containing NF-kB binding sites WO9842738-A1 Nucleotide sequence encoding Human cytostatin II WO9844109-A1 Human thymus receptor tyrosine kinase-related clone WO9844111-A1 T09276 cDNA clone H47991 WO9844112-A1 DNA encoding human chemokine beta-6 WO9844118-A1 Human immunoglobulin G (IgG) Fc coding region WO9845712-A2 Human EEGF genomic DNA WO9846746-A1 EDG-1-like G-protein coupled receptor WO9850549-A2 Nucleotide sequence encoding the human antimicrobial WO9851794-A1 protein cDNA clone W73681.nt which is related to GDNFR- WO9853069-A2 beta sequences Human tissue plasminogen activator-like protease WO9854199-A1 t-PALP DNA Human tumour necrosis factor receptor-like protein 8 WO9854201-A1 Human TNF receptor TR10 DNA WO9854202-A1 Gene No. 27 encoding human secreted protein WO9854206-A1 Polynucleotide fragment of gene 56 clone HE2OF09 WO9854963-A2 Human secreted protein gene 47 clone HOSCZ41 WO9856804-A1 Novel human tumor necrosis factor receptor TR9 WO9856892-A1 Human heregulin-like factor WO9857989-A1 Interferon Stimulating Protein And Uses Thereof WO9900412 Human cardiotrophin-like cytokine PCR 5'-primer #5 WO9900415-A1 Human NK-3 prostate specific gene-1 (NKX3.1) WO9900498-A1 Human secreted protein gene 10 clone HSKGO49 WO9901020-A2 Human secreted protein gene 122 clone HSVAQ28 WO9902546-A1 Histidine Kinase Two-component in Candida Albicans WO9902700 cDNA encoding interleukin-20 WO9903982-A1 Human secreted protein gene 51 clone HEBCM63 WO9903990-A1 Human secreted protein gene 73 WO9906423-A1 DNA encoding a human secreted protein WO9907891-A1 sequence of the human IgGFc region WO9909152-A1 Human secreted protein cDNA fragment containing WO9909155-A1 gene 33 Pancreas derived plasminogen activator inhibitor WO9909161-A1 protein Human nodal protein encoding DNA WO9909198-A1 Human IgG Fc coding region WO9910363-A1 Human follistatin-3 coding sequence fragment WO9910364-A1 HLMNX90R DNA encoding a human secreted protein WO9911293-A1 Interleukin-17 Receptor-like Protein WO9914240 EXPRESSION CONTROL SEQUENCES WO9916858 Human secreted protein cDNA fragment containing WO9918208-A1 gene 93 Human IgG Fc coding region WO9919339-A1 Human Tumor Necrosis Factor Receptor-like Proteins WO9920758 TR11, TR11SV1, and TR11SV2 Human chemokine alpha-6, designated HFCET92 WO9921575-A1 DNA encoding a human secreted protein WO9922243-A1 VEGI-alpha cDNA clone HEMFG66 WO9923105-A1 DNA encoding the human caspase-14 (ERICE) protein WO9923106-A1 Human IgG Fc coding region WO9924836-A1 Chemokine Alpha-5 WO9927078 Human IRAK-2 alpha and beta WO9927112-A1 Synferon, a Synthetic Type I Interferon WO9929862 Human Dendriac and Brainiac-3 WO9931116 110 Human Secreted Proteins WO9931117 Keratinocyte Growth Factor-2 Formulations WO9932135 36 Human Secreted Proteins WO9935158 Human Fk506 Binding Proteins WO9935160 Apoptosis Inducing Molecule II WO9935262 Human Ependymin WO9936565 67 Human Secreted Proteins WO9938881 Human Cystatin F WO9938882 45 Human Secreted Proteins WO9940100 Human Serine

Protease and Serpin Polypeptides WO9940183 Dendritic Enriched Secreted Lymphocyte Activation WO9940184 Molecule Therapeutic Uses of Keratinocyte Growth Factor-2 WO9941282 Apoptosis Inducing Molecule II and Methods of Use WO9942584 Human Secreted Proteins WO9943693 Human Secreted Proteins WO9946289 Angiogenic proteins and Uses Thereof WO9946364 Oxalyl-CoA decarboxylase ZA9403789-A Interferon Receptor HKAEF92 WO 99/62934 Cytokine Receptor Common Gamma Chain-like WO 99/47538 CTGF-4 WO 99/62927 Epithelial Specific Transcription Factor PDEF WO 00/06589 IL-21 and IL-22 WO 99/61617 Keratinocyte Derived Interferon WO 00/05371 FGFR-5 WO 00/24756 Hyaluronan-binding Protein PCT/US99/30462 12 Human Secreted Proteins WO 00/29435 TR12 WO 00/23572 Prostacyclin-stimulating factor/PGI2 WO 00/36105 Peptidoglycan Recognition Proteins WO 00/39327 Chemokine Beta-7 WO 00/28035 Brainiac-5 WO 00/39136 Chemokine Beta-10 PCT/US00/00296

US-PAT-NO: 6372212
DOCUMENT-IDENTIFIER: US 6372212 B1
TITLE: Chitinase materials and methods
DATE-ISSUED: April 16, 2002
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Gray; Patrick W.	Seattle	WA	N/A	N/A

US-CL-CURRENT: 424/94.61,435/209 ,536/23.2
ABSTRACT:

The present invention provides purified and isolated polynucleotide sequences encoding **human chitinase**. Also provided are materials and methods for the recombinant production of **human chitinase** products which are expected to be useful as products for treating fungal infections or for development of products useful for treating the same.

8 Claims, 0 Drawing figures
Exemplary Claim Number: 1

DATE FILED: June 16, 1997

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ABPL:
The present invention provides purified and isolated polynucleotide sequences encoding **human chitinase**. Also provided are materials and methods for the recombinant production of **human chitinase** products which are expected to be useful as products for treating fungal infections or for development of products useful for treating the same.

BSPR:
The present invention relates generally to **human chitinase** enzyme and more specifically to novel purified and isolated polynucleotides encoding **human chitinase,** to the chitinase products encoded by the polynucleotides, to materials and methods for the recombinant production of chitinase products and to antibody substances specific for the chitinase.

BSPR:
Escott et al., Infect. Immun., 63:4770-4773 (1995) demonstrated **chitinase enzymatic activity in human** leukocytes and in human serum. Overdijk et al., Glycobiology, 4:797-803 (1994) described isolation of a chitinase (4-methylumbelliferyl-tetra-N-acetylchitotetraoside hydrolase) from human serum and rat liver. Renkema et al., J. Biol. Chem., 270:2198-2202 (February 1995) prepared a **human chitotriosidase** from the spleen of a Gaucher disease patient. Their preparation exhibited chitinase activity and the article reports a small amount of amino acid sequence of the protein component of the preparation (22 amino terminal residues and 21 residues of a tryptic fragment). The function of **human chitinase** is also unknown, but a relationship with the pathophysiology of Gaucher disease is proposed in the article. A later publication by the same

group [Boot et al., J. Biol. Chem., 270(44):26252-26256 (November 1995)] describes the cloning of a human macrophage cDNA encoding a product that exhibits chitinase activity. The partial amino acid sequence reported by the group in their February 1995 article matches portions of the deduced amino acid sequence of the human macrophage cDNA product. See also International Patent Publication No. WO 96/40940.

BSPR:
In view of the increasing incidence of life-threatening fungal infection in immunocompromised individuals, there exists a need in the art to identify and isolate polynucleotide sequences encoding **human chitinases,** to develop materials and methods useful for the recombinant production of the enzyme, and to generate reagents for the detection of the chitinase in plasma.

BSPR:
The present invention provides novel purified and isolated polynucleotides (i.e., DNA and RNA, both sense and antisense strands) encoding **human chitinase** or fragments and analogs thereof; methods for the recombinant production of chitinase polypeptides, fragments and analogs thereof; purified and isolated chitinase polypeptide fragments and analogs; antibodies to such polypeptides, fragments and analogs; and pharmaceutical compositions comprising these polypeptides, fragments, analogs, or antibodies.

BSPR:
Specifically provided are: purified, isolated polynucleotides encoding the **human chitinase** amino acid sequence of SEQ ID NOS: 2 or 4, particularly amino acids 1 to 445 thereof; DNAs comprising the protein coding nucleotides of SEQ ID NOS: 1 or 3, particularly nucleotides 65 to 1402 of SEQ ID NO: 1 or nucleotides 90 to 1427 of SEQ ID NO: 3; purified, isolated polynucleotides comprising a polynucleotide sequence encoding the amino acid sequence of SEQ ID NOS: 14 or 15; purified, isolated polynucleotides encoding **human chitinase** selected from the group consisting of: (a) a double-stranded DNA comprising the protein coding portions of the sequence set out in either SEQ ID NO: 1 or SEQ ID NO: 3, (b) a DNA which hybridizes under stringent conditions to a non-coding strand of the DNA of (a), and (c) a DNA which, but for the redundancy of the genetic code, would hybridize under stringent conditions to a non-coding strand of DNA sequence of (a) or (b); vectors comprising such DNAs, particularly expression vectors wherein the DNA is operatively linked to an expression control DNA sequence; host cells stably transformed or transfected with such DNAs in a manner allowing the expression in said host cell of **human chitinase; a method for producing human chitinase** comprising culturing such host cells in a nutrient medium and isolating **human chitinase** from said host cell or said nutrient medium; purified, isolated polypeptides produced by this method; purified, isolated polypeptides comprising the **human chitinase** amino acid sequence of SEQ ID NOS: 2 or 4, particularly amino acids 1 to 445 thereof; **human chitinase** fragments lacking from 1 to about 72 C-terminal amino acid residues of mature **human chitinase, particularly the human chitinase** fragment of SEQ ID NO: 14; the **human chitinase** analog of SEQ ID NO: 15; hybridoma cell lines producing a monoclonal antibody that is specifically reactive with one of the above-described polypeptides; and monoclonal antibodies produced by such hybridomas.

BSPR:
Preferred DNA sequences of the invention include genomic and cDNA sequences as well as wholly or partially chemically synthesized DNA sequences. The nucleotide sequence of two human cDNAs encoding presumed allelic variants of **human chitinase**, and including noncoding 5' and 3' sequences, are set forth in SEQ ID NO: 1 and SEQ ID NO: 3. These DNA sequences and DNA sequences which hybridize to the noncoding strand thereof under standard stringent conditions or which would hybridize but for the redundancy of the genetic code, are contemplated by the invention. Preferred DNAs of the present invention comprise the **human chitinase** coding region (corresponding to nucleotides 2 to 1402 of SEQ ID NO: 1 or nucleotides 27 to 1427 of SEQ ID NO: 3), and the putative coding sequence of the mature, secreted **human chitinase** protein without its signal sequence (nucleotides 65 to 1402 of SEQ ID NO: 1, or nucleotides 90 to 1427 of SEQ ID NO: 3).

BSPR:
Two amino acid sequences for **human chitinase**(s) are set forth in SEQ ID NOS: 2 and 4. The sequence of SEQ ID NO: 2 is encoded by the nucleotide sequence of SEQ ID NO: 1, and SEQ ID NO: 4 is encoded by the nucleotide sequence of SEQ ID NO: 3. Preferred polynucleotides of the present invention include, in addition to those polynucleotides described above, polynucleotides that encode amino acids -21 to 445 of SEQ ID NO: 2 or SEQ ID NO: 4, and that differ from the polynucleotides described in the preceding paragraphs only due to the well-known degeneracy of the genetic code. Similarly, since twenty-one amino acids (positions -21 to -1) of SEQ ID NOS: 2 and 4 comprise a signal peptide that is cleaved to yield the mature **human chitinase** protein, preferred polynucleotides include those encoding polypeptides comprising amino acids 1 to 445 of SEQ ID NO: 2 or SEQ ID NO: 4.

BSPR:
Among the uses for the polynucleotides of the present invention is use as a hybridization probe, to identify and isolate genomic DNA encoding **human chitinase; to identify and isolate non-human** genes encoding proteins homologous to **human chitinase; to identify human** and non-**human proteins having similarity to human chitinase** (including those that may be involved in tissue remodeling); and to identify those cells which express **human chitinase** and the biological conditions under which this protein is expressed.

BSPR:
Chitinase products may be obtained as isolates from natural cell sources or may be chemically synthesized, but are preferably produced by recombinant procedures involving procaryotic or eucaryotic host cells of the invention. Chitinase products of the invention may be full length polypeptides, fragments or analogs thereof. Chitinase products having part or all of the amino acid sequence set out in SEQ ID NO: 2 or SEQ ID NO: 4 are contemplated. One preferred fragment which lacks the C-terminal seventy-two amino acid residues of the mature protein is set forth in SEQ ID NO: 14. It has been determined that these seventy-two C-terminal residues are not critical to chitinase enzymatic activity. Example 5 illustrates production of this C-terminal fragment; the introduction of a stop codon after the codon for amino acid 373 resulted in a recombinant chitinase fragment of about 39 kDa that retained similar specific activity when compared with full length recombinant **human**

chitinase.

BSPR:
The scientific value of the information contributed through the disclosures of DNA and amino acid sequences of the present invention is manifest. As one series of examples, knowledge of the sequence of a cDNA for chitinase makes possible the isolation by DNA/DNA hybridization of genomic DNA sequences encoding chitinase and chitinase expression control regulatory sequences such as promoters, operators and the like. DNA/DNA hybridization procedures carried out with DNA sequences of the invention under conditions of stringency standard in the art are likewise expected to allow the isolation of DNAs encoding **human allelic variants of chitinase, other structurally related human** proteins sharing one or more of the biochemical and/or immunological properties of **chitinase, and non-human species proteins homologous to chitinase**. The DNA sequence information provided by the present invention also makes possible the development, by homologous recombination or "knockout" strategies [see, e.g., Kapecchi, Science, 244: 1288-1292 (1989)], of rodents that fail to express a functional chitinase enzyme, overexpress chitinase enzyme, or express a variant chitinase enzyme. Polynucleotides of the invention when suitably labelled are useful in hybridization assays to detect the capacity of cells to synthesize chitinase. Polynucleotides of the invention may also be the basis for diagnostic methods useful for identifying a genetic alteration(s) in the chitinase locus that underlies a disease state or states. Also made available by the invention are anti-sense polynucleotides relevant to regulating expression of chitinase by those cells which ordinarily express the same.

BSPR:
Administration of chitinase preparations of the invention to mammalian subjects, especially humans, for the purpose of ameliorating disease states caused by chitin-containing parasites such as fungi is contemplated by the invention. Fungal infections (mycoses) such as candidiasis, aspergillosis, coccidioidomycosis, blastomycosis, paracoccidioidomycosis, histoplasmosis, cryptococcosis, chromoblastomycosis, sporotrichosis, mucormycosis, and the dermatophytoses can manifest as acute or chronic disease. Pathogenic fungi cause serious, often fatal disease in immunocompromised hosts. Cancer patients undergoing chemotherapy, immunosuppressed individuals, and HIV-infected individuals are susceptible to mycoses caused by Candida, Aspergillus, Pneumocystis carinii, and other fungi. Amphotericin B and fluconazole are useful therapeutics for fungal infections, but toxicity associated with these drugs causes serious adverse side effects that limit their usefulness. The mortality of systemic candidiasis is greater than 50% despite Amphotericin B treatment. Therefore, a need exists for agents that inhibit fungal growth in vivo; and such products may be used as single agents or in combination with currently approved, conventional anti-fungal compounds. Because growing fungi require chitin synthesis for survival, inhibition by recombinant **human chitinase** may be useful for limiting fungal infections in vivo. Animal models for fungal infection are illustrated below in Examples 8 through 14 and have been described in the art.

BSPR:
The **human chitinase** cDNA of the present invention was isolated from a macrophage cDNA library. Macrophages are known to be closely associated with rheumatoid arthritis lesions [Feldman et al., Cell, 85:307-310 (1996)], and

macrophage products such as TNF-.alpha. are implicated in disease progression. A protein with homology to **human chitinase,** C-gp39, has been detected in the synovium and cartilage of rheumatoid arthritis patients. While the natural substrate for **human chitinase** is probably chitin from pathogenic organisms, the enzyme may also exhibit activity on endogenous macromolecules which form the natural extraceflular matrix. For example, it has been suggested that hyaluronic acid, a major component of the extracellular matrix, contains a core of chitin oligomers. [Semino et al., Proc. Nat'l Acad. Sci., 93:4548-4553 (1996); Varki, Proc. Nat'l. Acad. Sci., 93:4523-4525 (1996).] Chitinase may therefore be involved in degradation of extracellular matrix in diseases such as rheumatoid arthritis. The role of chitinase may be determined by measuring chitinase levels and/or the effects of chitinase administration or chitinase inhibition in synovial fluid isolated from arthritic joints. Endogenous chitinase levels can be measured by enzymatic assay or with an antibody. Viscosity of synovial fluid can be measured before and after chitinase treatment; a decrease of viscosity associated with chitinase would be consistent with an endogenous chitinase substrate. Modulation of chitinase activity could thereby modulate the progression of joint destruction in rheumatoid arthritis.

BSPR:
Other aspects and advantages of the present invention will be understood upon consideration of the following illustrative examples. Example 1 describes the isolation of **human chitinase cDNA clones from a human** macrophage cDNA library. Example 2 addresses the pattern of **chitinase gene expression in various human** tissues. Example 3 describes the recombinant expression of the **human chitinase** gene in prokaryotic cells and purification of the resulting enzyme. Example 4 provides a protocol for the recombinant production of **human chitinase** in yeast. Example 5 describes the recombinant expression of the **human chitinase** gene in mammalian cells and purification of the resulting protein. Example 6 describes production of **human chitinase** polypeptide analogs by peptide synthesis or recombinant production methods. Example 7 provides a protocol for generating monoclonal antibodies that are specifically immunoreactive with **human chitinase**. Example 8 describes an assay for the measurement of chitinase catalytic activity. Example 9 addresses determination of the anti-fungal activity of **human chitinase** in vitro. Example 10 addresses determination of the anti-fungal activity of **human chitinase** in vivo in a mouse model, and Examples 11 through 14 address rabbit models of invasive aspergillosis, disseminated candidiasis, Candida ophthalmitis, and Candida endocarditis.

DEPR:
The nucleotide and deduced amino acid sequence of these cDNA clones were compared to sequences in nucleotide and peptide sequence databases to determine similarity to known genes. Sequence comparisons were performed by the BLAST Network Service of the National Center for Biotechnology Information using the alignment algorithm of Altschul et al., J. Mol. Biol., 215:403-410 (1990). Clone MO-911 exhibited significant homology to several different sequences, including mouse macrophage secretory protein YM-1 precursor (Genbank accession no. M94584), human cartilage gp-39 (Hakala et al., supra), oviductal glycoprotein from sheep, cow, and humans (DeSouza et al., supra), and chitinases from parasite (Oncocerca, Genbank accession no. U14639), wasp (Chelonus, Genbank accession no. U10422), plant (Nicotiana, Genbank accession

no. X771 11), and bacteria (Serratia, Genbank accession no. Z36295); its highest observed homology was to mammalian genes that encoded proteins with chitinase homology but no demonstrated chitinase activity. Further sequence analysis of MO-911 suggested that it contained a portion of the coding region for a **human chitinase** homolog.

DEPR:
The DNA sequence of clone pMO-218 (deposited on Jun. 7, 1996 under the terms of the Budapest Treaty with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md. 20852, U.S.A. under Accession No. 98077) is set forth in SEQ ID NO: 1, and the encoded amino acid sequence is set forth in SEQ ID NO: 2. MO-218 appeared to include the entire coding region of the **human chitinase** cDNA (nucleotides 2 to 1402 of SEQ ID NO: 1), which comprises a twenty-one amino acid putative signal sequence followed by 445 encoded amino acids (residues 1 to 445 of SEQ ID NO: 2). The twenty-two amino acids following the putative signal sequence exactly match the aminoterminal sequence of purified **human chitotriosidase** reported in Renkema et al., supra.: Renkema et al. also described a twenty-one amino acid sequence from a tryptic fragment of **human chitotriosidase** which corresponds exactly to residues 157 to 177 of MO-218 (SEQ ID NO: 2). Boot et al., supra, report the cloning of a **human chitotriosidase** cDNA which contains a coding sequence essentially identical to that of MO-218. The sequence of MO-218 differs from Boot et al. by an additional fourteen nucleotides at the 5' end and by a nucleotide change at nucleotide 330 in the coding region.

DEPR:
Northern blot analysis was performed to identify tissues in which the **human chitinase** is expressed. A multiple human tissue Northern blot (Clontech, Palo Alto Calif.) was hybridized with the entire coding region of MO-218 under standard stringent conditions (according to the Clontech laboratory manual). Greatest hybridization was observed to lung tissue (+++) and ovary (+++), with much smaller levels (+) in thymus and placenta. The size of the hybridizing mRNA was 2.0 kb for lung, ovary and thymus, which corresponds well with the size of the cloned cDNA (1.6 kb, or about 1.8 kb including the polyA tail). The size of the hybridizing placental mRNA was considerably smaller, at 1.3 kb. Chitinase hybridization was not observed in spleen, prostate, testes, small intestine, colon, peripheral blood leukocytes, heart, brain, liver, skeletal muscle, kidney, or pancreas. Chitinase expression in lung is consistent with a protective role against pathogenic organisms that contain chitin, since the lung represents the primary route of entry for fungal pathogens.

DEPR:
Transformants containing the resulting expression plasmid (pAraMO218) were induced with arabinose and grown at 37.degree. C. These transformants produced inclusion bodies containing a 39 kDa protein which was a truncated form of chitinase (engineered to contain 373 instead of 445 amino acids). This chitinase fragment contains four cysteine residues, while the full length chitinase contains ten cysteine residues. The inclusion bodies were separated from the E. coli culture and electrophoresed on SDS-PAGE. The 39 kDa band was transferred to a PVDF membrane and amino terminal sequenced. The majority (about two-thirds) of the material contained a sequence corresponding to the amino terminus of **human chitinase**. The remaining material corresponded to a

contaminating E. coli protein, porin. This recombinant chitinase preparation from E. coli was useful for producing polyclonal and monoclonal antibodies (described below in Example 7).

DEPR:
Exemplary protocols for the recombinant expression of **human chitinase** in yeast and for the purification of the resulting recombinant protein follow. The coding region of **human chitinase** is engineered into vectors for expression in Saccharomyces cerevisiae using either PCR or linker oligonucleotides designed to encode a fusion polypeptide containing a secretion mediating leader to the coding region for **human chitinase** corresponding to the amino terminus of the natural molecule. Secretion signal peptides include, e.g., SUC2 or equivalent leaders with a functional signal peptidase cleavage site, or pre-pro-alpha factor or other complex leader composed of a pre, or signal peptide, and a pro, or spacer region, exhibiting a KEX2 cleavage site. The DNA encoding the signal sequence can be obtained by oligonucleotide synthesis or by PCR. The DNA encoding the pre-pro-alpha factor leader is obtained by PCR using primers containing nucleotides 1 through 20 of the alpha mating factor gene and a primer complementary to nucleotides 255 through 235 of this gene [Kujan and Herskowitz, Cell, 30.933-943 (1982)). The pre-pro-alpha leader coding sequence and **human chitinase** coding sequence fragments are ligated into a plasmid containing the yeast alcohol dehydrogenase (ADH2) promoter, such that the promoter directs the expression of a fusion protein. As taught by Rose and Broach, [Meth. Enz., 185:234-279, D. Goeddel, ed., Academic Press, Inc., San Diego, Calif. (1990)], the vector further includes an ADH2 transcription terminator downstream of the cloning site, the yeast "2-micron" replication origin, a selectable marker, for example TRP1, CUP1 or LEU2 (or LEU2-d) or other equivalent gene, the yeast REP1 and REP2 genes, the E. coli beta lactamase gene, and an E. coli origin of replication. The beta-lactamase and TRP1 genes provide for selection in bacteria and yeast, respectively. The REP1 and REP2 genes encode proteins involved in plasmid copy number replication.

DEPR:
The DNA constructs described in the preceding paragraphs are transformed into yeast cells using a known method, e.g. lithium acetate treatment [Stearns et al., Meth. Enz., supra, pp. 280-297] or by equivalent methods. The ADH2 promoter is induced upon exhaustion of glucose in the growth media [Price et al., Gene, 55:287 (1987)]. The pre-pro-alpha sequence or other leader sequence effects secretion of the fusion protein, releasing the mature **human chitinase** peptide from the cells. The signal peptide leader is processed by signal peptidase or, in the case of pre-pro-alpha removal of the pro region, by the KEX2 protease Bfitter et al., Proc. Natl. Acad. Sci. USA, 81:5330-5334 (1984)].

DEPR:
The secreted recombinant **human chitinase** is purified from the yeast growth medium by, e.g., the methods used to purify chitinase from bacterial and mammalian cell supernatants (see Example 3 above and Example 5 below).

DEPR:
The MO-218 clone and the MO-13B clone, both of which contain full length **human chitinase** cDNA 3' to the CMV promoter of pRc/CMV, were isolated. A third plasmid, which corresponded to the same C-terminal fragment expressed in

bacterial cells in Example 3 above, was prepared as follows. The MO-218 plasmid was amplified by PCR using oligonucleotide primer 218-1 (CGCAAGCTTGAGAGCTCCGTTCCGCCACATGGTGCGGTCTGTGGCCTGG G, SEQ ID NO: 12), which
contains a Hind III site and nucleotides 2 through 23 of the MO-218 chitinase cDNA of SEQ ID NO: 1, and with complementary downstream primer T-END (GACTCTAGACTAGGTGCCTGAAGGCAAGTATG, SEQ ID NO: 13), which contains nucleotides
1164 through 1183 of MO-218, a stop codon and an Xbal site. The amplified DNA was purified by electrophoresis, digested with Xbal and HindIII, and cloned into the pRc/CMV vector (Invitrogen, San Diego, Calif.) previously cut with the same restriction enzymes. The junctions of the resulting clone was sequenced on a Model 373 (Applied Biosystems, Foster City, Calif.) and encoded the predicted engineered protein sequence, set forth in SEQ ID NO: 14.

DEPR:
Recombinant **human chitinase** was purified as follows. Five days after transfection of COS cells with the pRc/CMV-MO-13B plasmid, conditioned media from the culture was harvested and diluted with an equal volume of water. The diluted conditioned media was applied to a Q-Sepharose Fast Flow column (Pharmacia Biotech, Uppsala, Sweden) which was pre-equilibrated in 25 mM Tris, 10 mM sodium chloride, 1 mM EDTA, at pH 8.0. Approximately 95 % of the chitinase activity flowed through and did not bind to the column. This Q-Sepharose flow through was adjusted to 1.2 M ammonium sulfate and applied to a Butyl-Sepharose 4 Fast Flow column (Pharmacia) which was pre-equilibrated in 25 mM Tris, 1.2 M ammonium sulfate, 1 mM EDTA, at pH 8.0. Protein was eluted using a reverse gradient of 1.2 M to 0 M ammonium sulfate in 25 mM Tris, pH 8.0. A single absorbance peak at 280 nm corresponding to the chitinase activity peak was eluted at low salt. This material was greater than 85 % pure as determined by SDS-PAGE and contained approximately 60% of the chitinase activity. The protein was then concentrated and buffer exchanged into 20 mM Tris, 150 mM sodium chloride, at pH 8.0 using a 10,000 MWCO membrane (Ultrafree 10K, Millipore Corp., Bedford, Mass.). This preparation was then tested for enzymatic and anti-fungal activity in vitro as described in Examples 8 and 9 below. The recombinant preparation had a chitotriosidase activity of 90 nm/min per mg protein.

DEPR:
The supernatant from the pHDEF1/CTN.1 transfected CHO cells containing overexpressed recombinant **human chitinase** (rH-Chitinase) was purified as follows. In preparation for anion exchange chromatography, the supernatant was diluted 1:3 with 20 mM Tris, pH 7.0 (Buffer A). An anion exchange column, packed with Q-Sepharose Fast Flow Resin (Pharmacia Biotech Inc., Piscataway, N.J.), was equilibrated with Buffer A and loaded with 15L diluted supernatant per 1L resin. The rH-Chitinase was collected in the Flow Through from the Q-Sepharose column and adjusted to 5 % Polyethylene Glycol (PEG) 400 (Mallinckrodt Baker, Inc., Phillipsburg, N.J.), 30 mM sodium acetate, pH 4.3 in preparation for cation exchange chromatography. A cation exchange column, packed with CM-Sepharose Fast Flow Resin (Phannacia Biotech Inc., Piscataway, N.J.), was equilibrated with 30 mM sodium acetate, 5% PEG 400, pH 4.3 (Buffer B). The rH-Chitinase sample was loaded onto the CM-Sepharose column at 1 mg per mL resin, and rH-Chitinase was eluted from the column with 40 mM Tris, 5 % PEG 400, pH 7.5 (Buffer C). The rH-Chitinase sample was then prepared for

hydrophobic interaction chromatography by adding (NH.sub.4).sub.2 SO.sub.4 to 1.5M. A column packed with Macro-Prep Methyl H1C Support, (Bio-Rad Laboratories, Hercules, Calif.,) was equilibrated with 20 mM Tris, 5% PEG 400, pH 7.0 (Buffer D) containing 1.5M (NH.sub.4).sub.2 SO.sub.4. The rH-Chitinase sample was loaded onto the Macro-Prep Methyl column at 1 mg per mL resin. The column was washed with Buffer D containing 1.1 M (NH.sub.4).sub.2 SO.sub.4, and rH-Chitinase was eluted with Buffer D containing 0.2M (NH.sub.4).sub.2 SO.sub.4. The purified eluate was exchanged into 150 mM NaCl, 20 mM Tris, pH 7.0 (Buffer E) by membrane filtration.

DEPR:
Recombinant techniques such as those described in the preceding examples may be used to prepare **human chitinase** polypeptide analogs or fragments. More particularly, polynucleotides encoding **human chitinase** are modified to encode polypeptide analogs of interest using well-known techniques, e.g., site-directed mutagenesis and polymerase chain reaction. C-terminal and N-terminal deletions may also be prepared by, e.g., deleting the appropriate portion of the polynucleotide coding sequence. See generally Sambrook et al., supra, Chapter 15. The modified polynucleotides are expressed recombinantly, and the recombinant polypeptide analogs or fragments are purified as described in the preceding examples.

DEPR:
Residues critical for **human chitinase** activity are identified, e.g., by homology to other **chitinases and by substituting alanines for the native human chitinase** amino acid residues. Cysteines are often critical for the functional integrity of proteins because of their capacity to form disulfide bonds and restrict secondary structure. To determine whether any of the cysteines in **human chitinase** are critical for enzymatic activity, each cysteine is mutated individually to a serme.

DEPR:
A 39 kDa C-terminally truncated fragment of the mature **human chitinase** protein was prepared as described above in Examples 3 and 5 by introduction of a stop codon after the codon for amino acid 373. This 39 kDa fragment lacked seventy-two C-terminal amino acid residues of the mature protein, including six cysteines, yet retained similar specific enzymatic activity compared to the full length recombinant **human chitinase**. This result indicates that the missing seventy-two C-terminal residues, including the six cysteines, are not required for enzymatic activity.

DEPR:
Further C-terminal deletions may be prepared, e.g., by digesting the 3' end of the truncated **human chitinase** coding sequence described in Example 3 with exonuclease m for various amounts of time and then ligating the shortened coding sequence to plasmid DNA encoding stop codons in all three reading frames. N-terminal deletions are prepared in a similar manner by digesting the 5' end of the coding sequence and then ligating the digested fragments into a plasmid containing a promoter sequence and an initiating methionine immediately upstream of the promoter site. These N-terminal deletion analogs or fragments may also be expressed as fusion proteins.

DEPR:
Alternatively, **human chitinase** polypeptide analogs may also be prepared by full or partial chemical peptide synthesis using techniques known in the art. [See, e.g., synthesis of IL-8 in Clark-Lewis et al., J. Biol Chem., 266:23128-34 (1991); synthesis of IL-3 in Clarke-Lewis et al., Science, 231:134-139 (1986); and synthesis by ligation in Dawson et al., Science, 266:776-779 (1994).] Such synthetic methods also allow the selective introduction of novel, unnatural amino acids and other chemical modifications.

DEPR:
The biological activities, including enzymatic, anti-fungal, and extracellular matrix remodeling activities, of the **human chitinase** polypeptide analogs are assayed by art-recognized techniques, such as those described in Examples 8 to 14 below.

DEPR:
The following two protocols (multiple challenge or single shot immunizations) may be used to generate monoclonal antibodies to **human chitinase**. In the first protocol, a mouse is injected periodically with recombinant **human chitinase** (e.g., 10-20 .mu.g emulsified in Freund's Complete Adjuvant) obtained as described in any of Examples 3 through 6. The mouse is given a final pre-fusion boost of **human chitinase** in PBS, and four days later the mouse is sacrificed and its spleen removed. The spleen is placed in 10 ml serum-free RPMI 1640, and a single cell suspension is formed by grinding the spleen between the frosted ends of two glass microscope slides submerged in serum-free RPMI 1640, supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin, and 100 .mu.g/ml streptomycin (RPMI) (Gibco, Canada). The cell suspension is filtered through sterile 70-mesh Nitex cell strainer (Becton Dickinson, Parsippany, N.J.), and is washed twice by centrifuging at 200 g for 5 minutes and resuspending the pellet in 20 ml serum-free RPMI. Splenocytes taken from three naive Balb/c mice are prepared in a similar manner and used as a control. NS-1 myeloma cells, kept in log phase in RPMI with 11 % fetal bovine serum (FBS) (Hyclone Laboratories, Inc., Logan, Utah) for three days prior to fusion, are centrifuged at 200 g for 5 minutes, and the pellet is washed twice as described in the foregoing paragraph.

DEPR:
On days 2, 4, and 6, after the fusion, 100 .mu.l of medium is removed from the wells of the fusion plates and replaced with fresh medium. On day 8, the fusion is screened by EUISA, testing for the presence of mouse IgG binding to **human chitinase** as follows. Immulon 4 plates (Dynatech, Cambridge, MA) are coated for 2 hours at 37.degree. C. with 100 ng/well of **human chitinase** diluted in 25 mM Tris, pH 7.5. The coating solution is aspirated and 200 ul/well of blocking solution [0.5 % fish skin gelatin (Sigma) diluted in CMF-PBS] is added and incubated for 30 min. at 37.degree. C. Plates are washed three times with PBS with 0.05% Tween 20 (PBST) and 50 .mu.A culture supernatant is added. After incubation at 37.degree. C. for 30 minutes, and washing as above, 50 .mu.l of horseradish peroxidase conjugated goat anti-mouse IgG(fc) (Jackson ImmunoResearch, West Grove, Pa.) diluted 1:3500 in PBST is added. Plates are incubated as above, washed four times with PBST, and 100 .mu.L substrate, consisting of 1 mg/ml o-phenylene diamine (Sigma) and 0.1 .mu.l/ml 30% H.sub.2 O.sub.2 in 100 mM Citrate, pH 4.5, are added. The color reaction is stopped after 5 minutes with the addition of 50 .mu.l of 15 %

H.sub.2 SO.sub.4. A.sub.490 is read on a plate reader (Dynatech). Selected fusion wells are cloned twice by dilution into 96-well plates and visual scoring of the number of colonies/well after 5 days. The monoclonal antibodies produced by hybridomas are isotyped using the Isostrip system (Boehringer Mannheim, Indianapolis, Ind.).

DEPR:
Alternatively, a second protocol utilizing a single-shot intrasplenic immunization may be conducted generally according to Spitz, Methods Enzymol., 121:33-41 (1986). The spleen of the animal is exposed and injected with recombinant **human chitinase** (e.g., 10-20 .mu.g in PBS at a concentration of about 0.02% to 0.04%, with or without an aluminum adjuvant) obtained as described in any of Examples 3 through 6, after which the spleen is returned to the peritoneal cavity and the animal is stitched closed. Three days later, the mouse is sacrificed and its spleen removed. A spleen cell suspension is prepared, washed twice with RPMI 1640. supplemented with 3% fetal calf serum (FCS), and resuspended in 25 ml of the same medium. Myeloma cells (NS-O) are collected at logarithmic growth phase, washed once and added to the spleen cell suspension in a 50 ml tube, at a ratio of 3:1 or 2:1 (spleen cells:myeloma cells). The mixture is pelleted at about 450 g (1500 rpm), the supernatant aspirated, and the pellet loosened by tapping the tube. Fusion is performed at room temperature by adding 1 ml of polyethylene glycol (PEG) 1500 over 1 minute, with constant stirring. The mixture is incubated for another minute, then 1 ml of warm RPMI (30 to 37.degree. C.) is added over 1 minute followed by 5 ml RPMI over 3 minutes and another 10 ml RPMI over another 3 minutes. The cell suspension is centrifuged and resuspended in about 200 ml of HAT selective medium consisting of RPMI 1640 supplemented with 100 U/ml penicillin, 100 .mu.g/ml streptomycin, 20% FCS, 100 mM hypoxanthine, 0.4 mM aminopterin and 16 mM thymidine. The cell suspension is dispensed in 1 ml volumes into tissue culture plates and incubated at 37.degree. C. in a humid atmosphere with 5 % CO.sub.2 -95 % air for 8 to 10 days. Supernatants are aspirated and the cells are fed with 1 ml HAT medium per well, every 2 to 3 days according to cell growth. Supernatants of confluent wells are screened for specific antibodies and positive wells are cloned.

DEPR:
The **chitotriosidase activity of the recombinant human chitinase** produced in COS cells as described in Example SA was determined to be 90 nm/min per mg protein.

DEPR:
In a preliminary experiment, recombinant **human chitinase** was tested for inhibition of fungal growth in vitro. The two fungi Candida albicans and Aspergillus fumigatis are serious pathogens for immunocompromised patients. Both Candida and Aspergillus were grown in RPMI growth media at 37.degree. C. to approximately 10,000-50,000 colony forming units (CFU) per ml. Recombinant **human chitinase** (produced in COS cells as described in Example 5A) was added to cultures at 0, 2.8, 11.25, or 45 .mu.g/ml. After 24 hours, fungal growth was assessed by turbidity of cultures. Under these non-physiological conditions in this assay, all cultures appeared to grow at comparable rates, independent of chitinase concentration. The concentration of fungi tested, however, is much higher than the fungal burden seen during fungal infection in vivo. Different results may be obtained under different conditions, e.g., with a lower fungal burden, or when **human chitinase** is tested in combination with other anti-fungal

agents. Chitinase is also expected to be more effective in Wvo under physiological conditions.

DEPR:
In additional experiments, the anti-fungal activity of recombinant **human chitinase** (produced in COS cells as described in Example 5A) was evaluated in an agar diffusion assay, in a broth assay according to National Committee on Clinical Laboratory Standards, and in a cell wall inhibition assay according to Selitrennikoff, Antimicrob. Agents Chemother., 23:757-765 (1983).

DEPR:
In the agar diffusion assay, approximately 1.times.10.sup.6 cells/ml of Candida albicans (ATCC no. 90028) inoculated into 1.5% agar (RPMI 1640 media buffered with 2-(N-morpholino)propanesulfonic acid (MOPS), pH 7.0. A disk containing 50 .mu.g of the sample (A: recombinant **human chitinase,** B: buffer control, C: control protein, D: a bacterial lysate with chitinase activity, or a known anti-fungal agent) was placed on the agar, and the zone of growth inhibition was measured. Results are shown in Table 1 below.

DEPR:
In the broth assay, 50 .mu.g/ml of the sample (A: recombinant **human chitinase,** B: buffer control, C: control protein, D: a bacterial lysate with chitinase activity, or a known anti-fungal agent) was added with a certain concentration of the test fungal organism to RPMI 1640 media buffered with MOPS, pH 7.0. The samples were incubated at 35.degree. C., with shaking at 120 rpm, for 48 hours, and then growth was evaluated by measuring the turbidity of the suspension. The approximate concentrations of the test fungi were as follows: 2.5.times.10.sup.4 cells/ml of Candida albicans (ATCC no. 90028); 5.times.10.sup.4 cells/ml of Candida albicans-polyene resistant (ATCC no. 38247); 1 x 104 cells/ml of Aspergillus fumigatus (ATCC no. 16424); 1.times.10.sup.4 cells/ml of Neurospora crassa (ATCC no. 18889); and 1.times.10.sup.4 cells/ml of Saccharomyces cerevisiae (ATCC no. 26108). Results are shown in Table 2 below.

DEPR:
The os-1 whole cell assay, which identifies inhibitors of fungal cell wall biosynthesis, was conducted essentially according to Selitrennikoff, supra, using an inoculum of 1.5.times.10.sup.5 protoplasts/ml embedded in agar (Vogel's Medium N, 7.5% sorbitol, 1.5 % sucrose, 10 .mu.g/ml nicotiniamide and 1 % agar) incubated at 25.degree. C. for 72 hours. The cultures were monitored for changes in growth and morphology after disks containing 50 .mu.g of the sample (A: recombinant **human chitinase,** B: buffer control, C: control protein, D: a bacterial lysate with chitinase activity, or a known anti-fungal agent) were placed on the agar medium. The os-1 cell is a mutant strain of Neurospora crassa that grows as protoplasts without cell walls when incubated under certain conditions at 37.degree. C., but regenerates a cell wall under the appropriate conditions when the temperature is shifted to about 22.degree. C. Samples that inhibit growth are considered fungal growth inhibitors and samples that prevent cell wall regeneration, but do not kill the cells, are considered cell wall-specific inhibitors. Results are shown in Table 3 below.

DEPR:
The pharmacokinetics of recombinant **human chitinase** in mice were determined as

follows. Female Balb/c mice, 6-8 weeks old, were injected intravenously in the tail vein with 0.5 mg/kg, 5.0 mg/kg and 50 mg/kg recombinant **human chitinase**. For each dose, mice were terminally bled at 0.01, 0.25, 1, 8 and 24 hours after injection (2 animals were used per time point per dosage). Serum samples were then assayed for chitinase activity and concentration. Results are shown in Table 4 below.

DEPC:
Chitinase Gene Expression Pattern in Human Tissues

DEPC:
Production of Recombinant **Human Chitinase** in Bacterial Cells

DEPC:
Production of Recombinant **Human Chitinase** in Yeast Cells

DEPC:
Production of Recombinant **Human Chitinase** in Mammalian cells

DEPC:
Production of **Human Chitinase** Analogs and Fragments

DEPC:
Preparation of Monoclonal Antibodies to **Human Chitinase**

CLPR:
8. The method of claim 1 wherein the subject is suffering from a fungal infection involving a fungal species whose growth is not effectively inhibited by contact with **human chitinase** alone.

ORPL:
G.H. Renkema et al., "Purification and Characterization of **Human Chitotriosidase,** a Novel Member of the Chitinase Family of Proteins", J. Biol. Chem. 270 (5): 2198-2202, Feb. 1995.*

ORPL:
R.G. Bootet al., "Cloning of a cDNA Ecoding **Chitotriosidase, a Human Chitinase** Produced by Macrophages", J. Biol. Chem. 270 (44): 26252-26256, Nov. 1995.*

ORPL:
Argueso et al., "Effect of the Enzymes **Chitinase and Neuraminidase on the Structure of Human** Ocular Mucus," Investigative Ophthalmology & Visual Science, 36(4):S997 (Mar. 15, 1995) (Abstract 4615-596).

ORPL:
Boot et al., "Cloning of cDNA Encoding **Chitotriosidase, A Human Chitinase** Produced by Macrophage," J. Biol. Chem., 270(44):26252-26256 (Nov. 3, 1995).

ORPL:
Escott et al., "**Chitinase Activity in Human** Serum and Leukocytes," Infect. Immun., 63(12):4770-4773 (Dec., 1995).

ORPL:
Overdijk et al., "**Human Serum Contains a Chitinase:** Identification of an Enzyme, Formerly Described as 4-Methylumbelliferyl-tetra-N-Acetylchitotetraoside Hydrolase (MU-TACT Hydrolase)," Glycobiology, 4(6):797-803 (1994).

ORPL:
Renkema et al., "Purification and Characterization of **Human Chitotriosidase,** a Novel Member of the Chitinase Family of Proteins," J. Biol. Chem., 270(5):2198-2202 (Feb. 3, 1995).

ORPL:
Renkema et al., "Synthesis, sorting, and processing into distinct isoforms of **human macrophage chitotriosidase,**" Eur. J. Biochem., 244(2):279-285 (1997).

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INVENTOR-INFORMATION:

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ABSTRACT:
Methods for analyzing tumor cells, particularly bladder tumor cells employ gene expression analysis of samples. Gene expression patterns are formed and compared to reference patterns. Alternatively gene expression patterns are manipulated to exclude genes which are expressed in contaminating cell populations. Another alternative employs subtraction of the expression of genes which are expressed in contaminating cell types. These methods provide improved accuracy as well as alternative basis for analysis from diagnostic and prognostic tools currently available.

21 Claims, 24 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 15

DATE FILED: February 22, 2000

---------- KWIC ---------

DETL:
63 20 44 105 233 U29171_at Human casein kinase I delta "mRNA," complete cds 104 137 20 190 27 317 U29175_at 53 63 153 200 20 87 U29295_at Human neuronal pentraxin II (NPTX2) gene 20 20 20 20 20 57 U29343_at Human hyaluronan receptor (RHAMM) "mRNA," complete cds 20 109 20 20 128 44 U29463_s_at Human cytochrome b561 gene 52 38 36 80 166 62 U29589_at Human m3 muscarinic acetylcholine receptor (CHRM3) "gene," complete cds 44 41 20 26 127 20 U29607_at Human methionine aminopeptidase "mRNA," complete cds 41 276 227 240 20 241 U29615_at **Human chitotriosidase** precursor "mRNA," complete cds 33 101 20 20 176 20 U29656_at Human DR-nm23 "mRNA," complete cds 158 412 387 174 325 251 U29680_at Human A1 protein "mRNA," complete cds 43 139 20 44 112 20 U29700_at Human anti-mullerian hormone type II receptor precursor "gene," complete cds 110 178 110 176 113 26 U29725_at Human BMK1 alpha kinase "mRNA," complete cds 69 20 20 66 20 25 U29943_s_at Human ELAV-like neuronal protein-2 Hel-N2 "mRNA," complete cds 20 20 20 20 34 20 U29953_ma1_at Human pigment epithelium-derived factor gene, complete cds. 349 185 20 42 231 388 U30185_at Human orphan opioid receptor "mRNA," complete cds 20 99 20 20 20 20 U30245_at Human myelomonocytic specific protein (MNDA) "gene," 5' flanking sequence and complete exon 1 /gb=U30245/ntype=DNA /annot=exon 20 20 20 20 26 40 U30246_at Human bumetanide-sensitive Na-K-Cl cotransporter (NKCC1) "mRNA," complete cds 20 77 49 42 50 20 U30255_at Human phosphogluconate dehydrogenase (hPGDH) "gene," complete cds 503 115 20 119 20 209 U30313_at Human

diadenosine tetraphosphate "mRNA," complete cds. /gb=U0313 /ntype=RNA 20 20 20 20 20 20 U30521_at Human P311 HUM -3.1 "mRNA," complete cds 23 70 20 20 60 20 U30610_at Human CD94 protein "mRNA," complete cds 20 135 20 20 20 55 U30825_at Human splicing factor SRp30c "mRNA," complete cds 206 355 918 439 206 137 U30827_s_at Human splicing factor SRp40-3 (SRp40) "mRNA," complete cds 79 82 369 216 20 96 U30828_at Human splicing factor SRp55-2 (SRp55) "mRNA," complete cds 37 37 93 20 112 151 U30872_at Human mitosin "mRNA," complete cds 20 20 40 66 20 20 U30888_at Human tRNA-guanine transglyosylase "mRNA," complete cds 138 266 128 23 193 221 U30894_at Human N-sulphoglucosamine sulphohydrolase "mRNA," complete cds 20 145 164 167 317 390 U30930_at Human UDP-Galactose ceramide galactosyl transfease (CGT) "mRNA," compelte cds 45 75 28 45 59 147 U30998_at Human (nmd) "mRNA," 3'UTR. /gb=U30998 /ntype=RNA 20 20 57 20 142 153 U30999_at Human (memc) "mRNA," 3'UTR. /gb=U30999 /ntype=RNA 44 146 113 80 162 133 U31099_at Human DP prostanoid receptor (PTGDR) mRNA, partial cds. 20 20 20 20 20 20 U31116_at Human beta-sarcoglycan A3b "mRNA," complete cds 21 67 20 20 20 20 U31120_ma1_at Human interleukin-13 (IL-13) precursor gene, complete cds. 82 155 20 173 438 309 U31176_at Human hERV1 "mRNA," complete cds 79 163 26 130 259 279 U31201_cds1_at Human laminin gamma2 chain gene (LAMC2), exon 23 and flanking sequences, and complete cds. 20 20 20 20 44 22 U31201_cds2_s_at Human laminin gamma2 chain gene (LAMC2) 20 20 20 20 20 20 U31215_s_at Human metabotropic glutamate receptor 1 alpha (mGluR1alpha) "mRNA," complete cds 20 20 20 20 317 20 U31216_s_at Human metabotropic glutamate receptor 1 beta (mGluR1beta) "mRNA," complete cds 20 20 20 20 20 20 U31248_at Human zinc finger protein (ZNF174) "mRNA," complete cds 63 38 40 65 367 123 U31342_at Human nucleobindin gene 171 292 141 186 529 327 U31382_at Human G protein gamma-4 subunit "mRNA," complete cds 114 20 26 102 163 111 U31383_at Human G protein gamma-10 subunit "mRNA," complete cds 20 142 167 66 25 20 U31384_at Human G protein gamma-11 subunit "mRNA," complete cds 139 161 25 59 138 81 U31449_at Human intestinal and liver tetraspan membrane protein (il-TMP) "mRNA," complete cds 20 20 20 20 20 20 U31501_at Human fragile X mental retardation syndrome related protein (FXR2) "mRNA," complete cds 147 217 20 129 182 406 U31556_at Human transcription factor E2F-5 "mRNA," complete cds 21 20 67 68 93 37 U31628_at Human interleukin-15 receptor alpha chain precursor (IL 15RA) "mRNA," complete cds 93 20 20 133 294 288 U31799_at Human melanocyte protein Pmel 17 gene 20 20 20 20 101 60 U31814_at Human transcriptional regulator homolog RPD3 "mRNA," complete cds 42 113 164 110 20 21 U31875_at Human Hep27 protein mRNA, complete cds. 126 1358 2242 1444 822 1012 U31903_s_at Human CREB-RP (creb-rp) "mRNA," complete cds 97 144 311 176 610 209 U31929_s_at Human orphan nuclear receptor (DAX1) "gene," complete cds 67 117 149 66 368 135 U31930_at Human deoxyuridine nucleotidohydrolase "mRNA," complete cds 57 164 161 177 155 68 U31973_s_at Human phosphodiesterase A' subunit (PDE6C) "mRNA," complete cds 20 20 20 20 128 20 U31986_at Human cartilage-specific homeodomain protein Cart-1 "mRNA," complete cds 63 119 139 29 145 248 U32114_at Human caveolin-2 "mRNA," complete cds 49 20 31 41 163 37 U32315_at Human syntaxin 3 "mRNA," complete cds 43 20 36 55 80 82 U32324_at Human interleukin-11 receptor alpha chain "mRNA," complete cds 69 20 63 20 75 20 U32331_at Human RIG "mRNA," complete sequence 47 22 20 20 20 20 U32376_at Human channel associated protein os synapse (chapsyn-110) "mRNA," complete cds 20 33 20 20 20 20 U32439_at Human regulator of G-protein signaling similarity (RGS7) "mRNA," partial cds 22 20 20 20 191 102 U32499_s_at Human d3 dopamine receptor "mRNA," complete cds. 20 20 20 20 20 20 20 U32519_at Human GAP SH3 binding protein "mRNA," complete cds

93 79 80 87 160 211 U32576_ma1_at Human apoliproprotein apoC-IV (APOC4) gene, complete cds. 20 20 20 24 20 20 U32581_at Human lambda/iota-protein kinase C-interacting protein "mRNA," complete cds 20 20 20 20 20 20 U32645_at Human myeloid elf-1 like factor (MDF) "mRNA," complete cds 20 20 20 20 188 20 U32659_at Human IL-17 "mRNA," complete cds 20 26 20 27 127 31 U32674_s_at Human orphan receptor GPR9 (GPR9) "gene," partial cds 20 77 92 20 329 151 U32680_at Human CLN3 "mRNA," complete cds 20 49 20 99 302 135 U32849_at Human Hou "mRNA," complete cds 39 20 29 23 20 49 U32907_at Human p37NB "mRNA," complete cds 20 20 20 20 20 20 U32944_at Human cytoplasmic dynein light chain 1 (hdlc1) "mRNA," complete cds 743 290 526 524 20 189 U32986_s_at Human xeroderma pigmentosum group E UV-damaged DNA binding factor "mRNA," complete cds 110 218 298 231 20 90 U32989_at Human tryptophan oxygenase (TDO) "mRNA," complete cds 20 20 20 20 20 20 U33017_at Human signaling lymphocytic activation molecule (SLAM) "mRNA," complete cds 20 20 20 20 20 112 U33052_s_at Human "lipid-activated," protein kinase PRK2 "mRNA," complete cds 59 41 190 117 169 95 U33053_at Human lipid-activated protein kinase PRK1 "mRNA," complete cds 54 57 20 78 20 209 U33054_at Human G protein-coupled receptor kinase GRK4 "mRNA," alpha splice "variant," complete cds 20 20 20 20 20 20 U33147_at Human mammaglobin "mRNA," complete cds 20 20 28 20 20 151 U33202_s_at Human mdm2-D (mdm2) "mRNA," complete cds. /gb=U33202 /ntype=RNA 20 20 34 21 20 35 U33203_s_at Human mdm2-E (mdm2) "mRNA," complete cds. /gb=U33203 /ntype=RNA 20 20 20 20 20 20 U33267_at Human glycine receptor beta subunit (GLRB) "mRNA," complete cds 21 57 20 20 34 36 U33286_at Human chromosome segregation gene homolog CAS "mRNA," complete cds 68 83 194 85 99 155 U33317_ma1_at Human defensin 6 (HD-6) gene, complete cds

US-PAT-NO: 6303118
DOCUMENT-IDENTIFIER: US 6303118 B1
TITLE: **Human chitinase,** its recombinant production, its use for decomposing chitin, its use in therapy or prophylaxis against infection diseases
DATE-ISSUED: October 16, 2001
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
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US-CL-CURRENT: 424/94.61,435/209 ,536/23.2
ABSTRACT:
A new **human chitinase** having an amino acid sequence as shown in FIG. 1 or FIG. 2. Modified forms of it having a similar chitin-hydrolyzing activity, and antigenic peptides representing one of its epitopes. Recombinant production of the **human chitinase** by genetically engineered hosts or host cells. Recombinant nucleic acid encoding it, and **human chitinase**-specific oligonucleotides. Use for therapeutic or prophylactic treatment of humans against infection by chitin-containing pathogens, or for decomposing chitin, e.g. from chitin-based articles. Antibodies binding to the **human chitinase**. Diagnostic test kits comprising the **human chitinase, its antigenic peptides, human chitinase** antibodies, recombinant nucleic acid or oligonucleotides.
6 Claims, 5 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 5

DATE FILED: June 30, 1999

---------- KWIC ---------

TTL:
Human chitinase, its recombinant production, its use for decomposing chitin, its use in therapy or prophylaxis against infection diseases

ABPL:
A new **human chitinase** having an amino acid sequence as shown in FIG. 1 or FIG. 2. Modified forms of it having a similar chitin-hydrolyzing activity, and antigenic peptides representing one of its epitopes. Recombinant production of the **human chitinase** by genetically engineered hosts or host cells. Recombinant nucleic acid encoding it, and **human chitinase**-specific oligonucleotides. Use for therapeutic or prophylactic treatment of humans against infection by chitin-containing pathogens, or for decomposing chitin, e.g. from chitin-based articles. Antibodies binding to the **human chitinase**. Diagnostic test kits comprising the **human chitinase, its antigenic peptides, human chitinase** antibodies, recombinant nucleic acid or oligonucleotides.

BSPR:
A **human chitinase,** its recombinant production, its use for decomposing chitin,

its use in therapy or prophylaxis against infection diseases.

BSPR:
Secondly, the use of chitinases from pathogenic organisms as a vaccine may result in unforeseen harmful side-effects. It cannot be excluded that fragments of such chitinases share homology with endogenous proteins and that an undesired immune response is elicited. This may in fact be more than a theoretical problem because of the strong homology between **human chitinase** and chitinases from other species (see below).

BSPR:
Given the limitations of current approaches to tackle chitin-containing pathogens, a novel approach is here proposed to solve the problem that constitutes a major threat to the welfare of man. The approach is based on the use of a recently identified **human chitinase,** which can be produced by recombinant DNA technology (biotechnology), as a safe and effective agent against chitin-containing pathogens, i.e. for intervention of infectious diseases caused by chitin-containing pathogens. The conception of the approach and its further development is described below.

BSPR:
The subject invention provides a substantially isolated or purified **chitinase, said chitinase being a human chitinase** having an amino acid sequence essentially corresponding to the amino acid sequence shown in FIG. 1 (SEQ ID NO:4) or the amino acid sequence shown in FIG. 2 (SEQ ID NO:6), or being a modified form of said **human chitinase** having a substantially similar chitin-hydrolyzing activity. It is preferred that this new **human chitinase** is produced by a genetically engineered host cell and isolated from said host cell or medium in which said host cell is cultured, wherein the amino acid sequence of the enzyme is encoded by a nucleotide sequence essentially corresponding to the nucleotide sequence shown in FIG. 1 (SEQ ID NO:3) or the nucleotide sequence shown in FIG. 2 (SEQ ID NO:5). The subject invention particularly includes a chitinase having an amino acid sequence essentially corresponding to the amino acid sequence shown in FIG. 1 (SEQ ID NO:4) and having a molecular weight of about 50 kDa, and a chitinase having an amino acid sequence essentially corresponding to the amino acid sequence shown in FIG. 2 (SEQ ID NO:6) and having a molecular weight of about 39 kDa.

BSPR:
The phrase "essentially corresponding to" intends to allow for small sequence variations, such as the naturally occurring variations which do not significantly affect the activity of the enzyme. Some amino acids of the **human chitinase** sequence may be replaced by others, or be deleted, without thereby significantly affecting the function, activity and tolerability of the enzyme, and may sometimes even improve one characteristic or the overall properties of the enzyme. Generally, such sequence variations will be quite limited, say to about less than 30%, more often less than 20% or even less than 10% of all amino acids, i.e. the variants will generally have a high homology of above 70%, more often above 80% or even above 90%, compared to the sequences shown in FIGS. 1 and 2 (SEQ ID NO:4 and 6). All have in common the functional characteristic of chitinase activity, which can be measured for typical chitinase substrates, such as 4-methylumbelliferyl-chitotrioside.

BSPR:
The phrase "a modified form of said **human chitinase** having a substantially similar chitin-hydrolyzing activity" intends to embrace variants whose amino acid sequence differs significantly from the sequences shown in FIGS. 1 and 2 (SEQ ID NO:4 and 6) but which yet have a similar chitinase activity. Such modified forms having similar or even improved properties could be designed on the basis of the module or domain structure of the **human chitinase,** such as constructs lacking a domain which is not required or even disadvantageous for activity, and constructs containing two or more copies of a domain whose amplified presence is desirable.

BSPR:
The phrase "having a substantially similar chitin-hydrolyzing activity" intends to set the minimum requirement of having an at least equivalent **chitinase activity compared to the human chitinases** shown in FIGS. 1 and 2 (SEQ ID NO:3-6). "Equivalent" refers to equivalency in substrate range, i.e. qualitatively, and to equivalency in activity value, i.e. quantitatively.

BSPR:
The subject invention furthermore provides a pharmaceutical composition comprising the new **human chitinase** as defined herein and a pharmaceutically acceptable carrier or diluent, more in particular a pharmaceutical composition for therapeutic or prophylactic treatment of a human individual against infection by a chitin-containing pathogen, comprising a therapeutically or prophylactically effective amount of the new **human chitinase** and a pharmaceutically acceptable carrier or diluent. Preferably the pharmaceutical composition further comprises a therapeutically or prophylactically effective amount of a human .beta.-1,3-glucanase.

BSPR:
The invention also provides non-pharmaceutical compositions comprising the new **human chitinase** and a carrier or diluent. For example, such composition may be a medium for culturing cells, in particular human cells, or be a cosmetic (e.g. body lotion), dental (e.g. tooth paste, mouth rinse) or food product (e.g. milk, cheese and other dairy products).

BSPR:
Furthermore, this invention provides chitin-based articles of manufacture comprising a chitin-hydrolyzing amount of the new **human chitinase**. E.g., the chitin-based article of manufacture may be a drug-containing drug carrier or implant for controlled drug release, or a transient functional implant.

BSPR:
This invention also provides a method of therapeutic or prophylactic treatment of a human individual against infection by a chitin-containing pathogen, comprising administering to said human individual a pharmaceutical composition comprising a therapeutically or prophylactically effective amount of the new **human chitinase**.

BSPR:
The subject invention also provides a process for preparing a **human chitinase** having an amino acid sequence essentially corresponding to the amino acid

sequence shown in FIG. 1 (SEQ ID NO:4) or the amino acid sequence shown in FIG. 2 (SEQ ID NO:6), or a modified form of said **human chitinase** having a substantially similar chitin-hydrolyzing activity, comprising growing a genetically engineered host or host cell capable of producing said **human chitinase** or modified form thereof and isolating the chitinase produced from said host or host cell or from medium in which said host cell is cultured. In this process, preferably the amino acid sequence of said chitinase is encoded by a nucleotide sequence essentially corresponding to the nucleotide sequence shown in FIG. 1 (SEQ ID NO:3) or the nucleotide sequence shown in FIG. 2 (SEQ ID NO:5).

BSPR:
The invention also provides a genetically engineered host cell capable of producing a **human chitinase** having an amino acid sequence essentially corresponding to the amino acid sequence shown in FIG. 1 (SEQ ID NO:4) or the amino acid sequence shown in FIG. 2 (SEQ ID NO:6), or a modified form of said **human chitinase** having a substantially similar chitin-hydrolyzing activity.

BSPR:
The invention also embraces oligonucleotides of at least about 8 nucleotides having a nucleotide sequence corresponding to, or complementary to, a nucleotide sequence shown in FIG. 1 (SEQ ID NO:3) or a nucleotide sequence shown in FIG. 2 (SEQ ID NO:5) and being capable of binding by hybridisation under stringent (i.e. requiring about complete complementarity) hybridisation conditions to nucleic acid coding for the new **human chitinase**. Such oligonucleotides can be useful for different purposes, e.g. as a primer for use in nucleic acid amplification methods such as PCR, NASBA etc., or as a probe in hybridisation analysis. The length will usually depend on the intended use. When used as a primer, the length will normally be between 12, preferably 15, and 25, preferably 20 nucleotides. When used as a probe, the length will usually be somewhat higher, e.g. from about 15 or 20 up to about 40 or 50 nucleotides, or even up to the complete length of the coding sequence.

BSPR:
Similarly, this invention furthermore embraces peptides of at least about 8 amino acid residues having an amino acid sequence derived from the amino acid sequence shown in FIG. 1 (SEQ ID NO:4) or the amino acid sequence shown in FIG. 2 (SEQ ID NO:6) and representing or mimicking an epitope of the new **human chitinase,** in particular those having an amino acid sequence corresponding to an amino acid sequence shown in FIG. 1 (SEQ ID NO:4) or an amino acid sequence shown in FIG. 2 (SEQ ID NO:6) and having antigenicity. Usually, such peptides will have a length of at least about 10, or even at least about 15 amino acid residues, and up to about 40, preferably up to about 30 amino acid residues. Said peptides can be used for diagnostic purposes, or in immunization protocols to raise **human chitinase**-specific antibodies.

BSPR:
The invention also embraces antibodies capable of binding to the new **human chitinase,** especially monoclonal antibodies. Such antibodies can be used for many purposes, for example for isolating and/or purifying (e.g. by affinity chromatography) the **human chitinase,** or for diagnostic purposes.

BSPR:
The subject invention furthermore provides a diagnostic kit comprising such a **human chitinase-binding antibody, or a human chitinase** peptide as defined above, or the new **human chitinase** itself as defined herein, together with a conventional component of diagnostic kits for detecting an antigen or an antibody; and a diagnostic kit comprising a **human chitinase**-specific oligo-nucleotide or recombinant **human chitinase**-encoding nucleic acid as defined herein, together with a conventional component of diagnostic kits for detecting a nucleic acid.

BSPR:
Furthermore, the subject invention provides a method of decomposing chitin comprising contacting said chitin with the new **human chitinase** under chitin-hydrolyzing conditions.

DEPR:
The fact that man is continuously exposed to chitin (or chitin-containing organisms) strongly suggests that man should also have the ability to degrade this material. A gradual, presumably lysosomal, accumulation of chitin would otherwise inevitably occur in life, e.g. in alveolar macrophages that are in continuous contact with chitin-containing organisms. However, such storage of chitin has never been noted. This prompted us to search for the occurrence of a **chitinase activity in human** macrophages. Indeed, as is documented below, we were able to demonstrate that, in contrast to previous believes, macrophages can produce a chitinase similar in properties to enzyme encountered in other non-mammalian organisms (17,18). The enzyme is highly capable of hydrolyzing chitin and also shows other common characteristics of chitinases. Based on the substrate initially used in the identification of the novel enzyme, i.e. 4-methylumbelliferyl-chitotrioside, the **human chitinase** has been named chitotriosidase (17).

DEPR:
In order to use the **human chitinase** (chitotriosidase) as a (pharmaceutical) agent against chitin-containing organisms in vivo, a number of conditions have to be fulfilled.

DEPR:
In order to be useful as an agent against chitin-containing pathogens chitotriosidase has furthermore to be available in large quantities in a uniform state. There are no ubiquitous, natural sources for the isolation of the **human chitinase**. The mounts of enzyme in urine and placentas are low. This led us to attempt to isolate cDNA encoding chitotriosidase. Due to the specific expression of the chitotriosidase gene in macrophages, all tested cDNA libraries from other cell types were found to be negative for chitotriosidase cDNA. However, a constructed cDNA library from mRNA of long-term cultured macrophages that secreted massive amounts of chitotriosidase activity proved to be extremely rich in cDNAs encoding chitotriosidase, (0.1% of total cDNA). Two distinct cDNAs were in this manner identified and cloned.

DEPR:
The findings suggest that large scale recombinant production of both forms of **human chitotriosidase** using conventional techniques should be feasible.

Moreover, it seems likely that not only production of the human enzyme in eukaryotic cells, but even in prokaryotes might be possible, since highly homologous proteins are endogenously produced by some of these organisms, e.g. Serratia marcescens. A procedure for the purification of chitotriosidase has been successfully developed (18; and below) It therefore will be possible to obtain large amounts of both recombinant **human chitotriosidases** in a pure and uniform state suitable for administration to man.

DEPR:
The 39 kDa chitozyme is not a glycosylated protein, so its production in prokaryotic cells should certainly be feasible. Bacteria which produce and secrete highly homologous chitinases should in principle be able to secrete correctly folded **human chitotriosidase** in their exoplasmic space, provided that a correct leader sequence is used. Alternatively, it could be considered to use yeast cells for the production of recombinant chitotriosidase, at least the 39 kDa chitozyme. It can so far not be excluded, however, that also 50 kDa chitotriosidase can be produced, not only in higher eukaryotes, but also in lower eukaryotes or even in prokaryotes.

DEPR:
A prerequisite for the intravenous application of the chitinase is insight in its clearance. In the blood stream the most predominant isozyme is the 50 kDa protein. In tissue predominantly a 39 kDa isozyme is encountered. This appears to be formed by uptake of 50 kDa protein followed by proteolytic cleavage to a 39 kDa form that is remarkably stable in the lysosomal environment. Experiments in rats suggest that the half life of recombinant 50 kDa chitotriosidase in the circulation is somewhat longer than that of the 39 kDa enzyme. Clearance is not a very rapid process as monitored by the disappearance of activity of **human chitotriosidase** in the blood stream of intravenously injected rats, the half life being about one hour. Only minor amounts of chitotriosidase are daily excreted into the urine. It is conceivable that some enzyme is efficiently recaptured by proximal tube epithelial cells since kidney is found to be extremely rich in `lysosomally processed` 39 kDa enzyme. The observations so far suggest that intravenous administration can lead to a high level of **human chitinase** activity in the circulation for a prolonged period of time, allowing enzyme to reach various tissue locations.

DEPR:
A. Use of a cocktail of recombinant **human chitinase** and .beta.-1,3-glucanase

DEPR:
It is well documented that both in plants and fish chitinases play an important role in resistance against fungal infections. In plants, chitinases act synergistically with .beta.-1,3glucanases since the cell walls of fungi are composed of a mixture of chitin and .beta.glucan fibrils (15). At present it is believed that man is not capable of producing a chitinase nor a .beta.-glucanase. However, it was noted that long-term cultured macrophages are not only able to secrete a chitinolytic enzyme but also an enzyme active against dye-labeled .beta.-glucan. We therefore propose that analogous to the situation in plants a mixture of **human chitinase** and .beta.-glucanase could be a more powerful anti-fungal agent than one of these enzymes alone. Isolation of the .beta.-glucanase produced by long-term cultured macrophages and

subsequent cloning of corresponding cDNA, should result in the availability of recombinant human .beta.-glucanase for this purpose.

DEPR:
B. Use of modified recombinant **human chitinase**

DEPR:
It cannot be excluded that a deficiency in chitotriosidase may be associated with some disadvantage. For example, the resistance against chitin-containing pathogens could be reduced and lysosomal degradation of chitin in phagocytes could be impaired, resulting in abnormal behaviour of the cells. Further research is required to establish whether a chitotriosidase deficiency is indeed associated with some risks. If this proofs to be the case, prophylactic administration of **human chitotriosidase** to deficient individuals could be considered.

DEPR:
The availability of a **human chitinase** could be also exploited as a tool to degrade injected or implanted chitinbased structures for medical purposes.

DEPR:
For example, drugs could be incorporated in chitin based capsules (`chitosomes`). The concomitant presence of well defined amounts of **human chitinase** in the capsule could ensure a controlled release of drugs. A slow but gradual release of drug could particularly be envisioned when it is trapped in a chitin matrix. The use of the human enzyme in such a system would result in ultimate destruction of the chitin-based capsule and not elicit an immunological response. The drugs used in such a system could vary from small compounds to proteins and DNA fragments for the purpose of enzyme and gene therapy. Chitin (or analogues) is already employed as a carrier for drugs (20).

DEPR:
Finally, recombinant **human chitotriosidase** (or a cocktail with .beta.-1,3-glucanase) may be used as an additive in tooth paste and body lotions in order to prevent fungal infections.

DEPR:
Cloning and Composition of cDNAs Encoding **Human Chitotriosidases**

DEPR:
In order to clone cDNA encoding **human chitotriosidase** the following strategy was used. Chitotriosidase was purified from spleen of a type 1 Gaucher disease patient since this organ is extremely rich in chitotriosidase activity (18). The N-terminal amino acid sequence of chitotriosidase was determined and this information was used for cloning chitotriosidase cDNA. Firstly, the established N-terminal amino acid sequence of chitotriosidase (18) was used to design a degenerate sense oligonucleotide: 5'-TGYTAYTTYACNAAYTGGGC-3'(SEQ ID NO:1). Secondly, a degenerate anti-sense nucleotide was designed based on the highly conserved domain among chitinases that is presumed to be an essential part of the catalytic center: 5'-CCARTCIARRTYIACICCRTCRAA-3' (SEQ ID NO:2).

DEPR:

These oligonucleotides were used to amplify a DNA fragment by RT-PCR. For this purpose, total RNA had been isolated from long-term cultured macrophages that secreted large amounts of chitotriosidase activity. First strand cDNA synthesis was performed using SuperScript TM RNAse H, reverse transcriptase and oligo dT. After alkaline hydrolysis, the cDNA was precipitated with ethanol and used as template. PCR was performed using standard conditions. The DNA fragment obtained by RT-PCR was of the expected size (on the basis of homology with members of the chitinase family). The fragment was purified, treated with T4 DNA polymerase and cloned into the HindII site of the plasmid vector pUC19. Determination of its sequence using the dideoxy-nucleotide chain termination method revealed that the fragment was in complete accordance with the known N-terminal amino acid sequence of purified **human chitotriosidase**, allowing its use as a probe to identify a full length chitotriosidase cDNA.

DEPR:
The nucleotide sequence (SEQ ID NO:3) of the cDNA clone chi.50 shows an open reading frame starting with an ATG at position 13 and ending with a TGA codon at position 1410 (see FIG.1). The open reading frame encodes a protein with a characteristic N-terminal ER signal peptide, immediately followed by the N-terminal sequence established for the chitotriosidase protein. The cDNA sequence does not indicate the presence of potential N-linked glycosylation sites, which is consistent with the absence of N-linked glycans in isolated chitotriosidase. The predicted protein, after removal of the signal sequence, has a length of 445 amino acids and a calculated molecular mass of 49 kDa. Metabolic labelling experiments with cultured macrophages revealed that these cells predominantly synthesize and secrete a chitotriosidase protein with apparent molecular mass of 50 kDa with polyacrylamide gel electrophoresis in the presence of sodium dodecylsulphate at reducing conditions. The predicted C-terminal part of 50 kDa **human chitotriosidase** is rich in serine residues of which theoretically some might be O-linked glycosylated. The occurrence of this type of glycans in 50 kDa **human chitotriosidase** has so far not been excluded or confirmed.

DEPR:
A search of the EMBL and GenBank databases revealed significant homology between the two **human chitotriosidases** and a group of chitinases and related proteins from different species. All the homologous proteins belong to the so called `chitinase protein family` (18,19).

DEPR:
The predicted C-terminal part of 50 kDa **human chitotriosidase** shows only homology with two chitinases from Manduca sexta and Brugia malayi, respectively. In the case of the latter enzyme O-linked glycosylation has been reported (12).

DEPR:
The chitotriosidase produced by COS cells was analysed by immunotitration with a rabbit antiserum against **human chitotriosidase**. This antiserum is capable of inhibiting **human chitotriosidase** in its enzymatic activity. FIG. 5 shows that chitotriosidase is inactivated by the antiserum in an identical manner to isolated splenic chitotriosidase. This finding suggests that the enzymatic activity per amount of antigen is similar in the case of the two recombinant chitotriosidases and the splenic enzyme.

DEPR:
Chitin azure (Sigma) suspended in citrate/phosphate buffer (pH 5.2) at a final concentration of 10 mg/ml was used to monitor chitinase activity. Chitin degradation was detected spectrophotometrically at 550 nm by determination of release of soluble azure (18). Chitinase from Serratia marcescens (Sigma) was used as control. When related to the hydrolysis of 4-methylumbelliferyl-chitotrioside, the **chitinase activity of human chitotriosidase** was comparable to that of the bacterial chitinase. See for example ref.18.

DEPR:
No significant activity of **human chitotriosidase** towards a cell wall suspension of Micrococcus lysodeikticus was detectable, suggesting that the enzymes lack lysozyme activity.

DEPR:
To test whether **human chitotriosidase** can exert an anti-fungal action, a chitinous fungus (Mucor mucedo) was grown on plates (containing malt extract, peptone, glucose and agar) under a Cellophane membrane in order to keep the hyphae flat against the agar surface (see ref.16). Individual sectors were cut out and mounted on microscope slides. Purified chitozyme 50 and chitozyme 39 were dialysed against 0.15 M sodium chloride. Samples of enzyme-containing solutions, and 0.15 M NaCl were pipetted on the hyphal tips. Microscopical analysis revealed that application of enzyme resulted in immediate cessation of hyphal growth, followed by a distorted morphological appearance. Application of saline had no effect. Negative effects on hyphal growth were detectable using chitozyme solutions with a concentration of enzyme as little as 0.005 mg/ml.

DEPR:
The proteins are: **human chitotriosidase** (SEQ ID NO:7); a chitinase from the virus Autographa californica (GenBank L22858(SEQ ID NO:8)); a chitinase from the tobacco hornworm Manduca sexta (GenBank U02270(SEQ ID NO:7)); an endochitinase from the nematode Brugia malayi (Genbank M73689(SEQ ID NO:9)); a human oviductal glycoprotein (GenBank U09550(SEQ ID NO:10)); HCgp-39, a human glycoprotein produced by chondrocytes and synovial cells (GenBank M80927(SEQ ID NO:11)); YM-1, a secretory protein of activated mouse macrophages (Pir S27879(SEQ ID NO:12)); a chitinase from the fungus Aphanocladium album (SwissProt P32470(SEQ ID NO:13)); a chitinase from the filamentous fungus Trichoderma harzianum (GenBank L14614(SEQ ID NO:14)); chitinase A1 from the prokaryote Bacillus circulans (SwissProt P20533(SEQ ID NO:15)); and a class V chitinase from the plant Nicotiana tabacum (GenBank X77110(SEQ ID NO:16)). Residues identical to chitotriosidase are indicated by the inverted characters. The proteins HCgp-39 and YM-1 are supposed to be not chitinolytic.

DEPR:
Preparations containing either purified 39 kDa splenic chitotriosidase ( ), or 50 kDa chitozyme produced by COS cells transfected with chi.50 cDNA ( ), or 39 kDa chitozyme produced by COS cells transfected with chi.39 cDNA were incubated for 1 hour at room temperature in phosphate buffered saline with different amounts of rabbit (anti-**human splenic chitotriosidase**) antiserum.

DEPC:
Recombinant Production of **Human Chitotriosidases**

DEPU:
18. Renkema, G. H., Boot, R. G., Muijsers, A. O., Donker-Koopman, W. E., Aerts, J. M. F. G. (1995), J. Biol. Chem. 270, 2198-2202. Purification and characterization of **human chitotriosidase,** a novel member of the chitinase family of proteins.

CLPR:
1. A method of therapeutic or prophylactic treatment of a human individual against infection by a chitin-containing pathogen, comprising administering to said human individual a pharmaceutical composition comprising a substantially isolated or purified chitinase and a pharmaceutically acceptable carrier or diluent, said **chitinase being a human chitinase** having the amino acid sequence of SEQ. ID NO:4 or SEQ. ID NO:6 or a **human chitinase** having chitinase activity, which is encoded by a nucleic acid which hybridizes to SEQ. ID NO:3 or SEQ. ID NO:5 at 65.degree. C., in 1 mM EDTA, 0.5 M sodium hydrogen phosphate (pH 7.2) containing 7% (w/v) SDS.

ORPL:
B. Overdijk et al., "**Human Serun Contains a Chitinase:** Identification of an Enzyme, Formerly Described as 4-Methylumbelliferyl-tetra-N-Acetylchitotetraoside Hydrolase (MU-Tact hydrolase)", Glycobiology 4(6): 797-803, 1994.*

US-PAT-NO: 6221591
DOCUMENT-IDENTIFIER: US 6221591 B1
TITLE: Determination of a genetic risk factor for infection and other diseases, and detection of activated phagocytes
DATE-ISSUED: April 24, 2001
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Aerts; J. M. F. G.	Abcoude	N/A	N/A	NLX

US-CL-CURRENT: 435/6,435/201 ,536/23.1 ,536/24.3
ABSTRACT:

It is shown that a defect in the chitotriosidase gene is a risk factor with respect to susceptibility for infectious diseases with chitin-containing pathogens and the development of (rheumatoid) arthritis. The molecular basis of the relatively common chitotriosidase deficiency is a 24 bp duplication in the chitotriosidase gene. A convenient method allowing analysis of chitotriosidase genotype and subsequent determination of increased risk has been developed. Chitotriosidase is shown to be selectively secreted by macrophages upon specific activation and excreted by neutrophils by release of specific granules upon an appropriate stimulus. It has been shown that the measurement of plasma chitotriosidase activity can be successfully used for diagnosis of specific disorders and monitoring of efficacy of therapeutic interventions, at least in combination with information on the chitotriosidase genotype status of an individual.

20 Claims, 22 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 19

DATE FILED: September 18, 1998

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BSPR:

Determination of the chitotriosidase genotype status of an individual is not only crucial for interpretation of enzyme activity levels in relation to diagnosis or monitoring of therapeutic intervention. Moreover, it is of great value for another reason. Infections with chitin-containing pathogens are a serious threat to mankind (for an extensive description see U.S. patent application Ser. No. 08/486,839). Most pathogens, with the exception of bacteria and viruses, have chitin as essential structural component in their coatings. It is known that chitinases in plants play an important role in defense against chitin-containing fungi [3]. Until recently it has generally been thought that vertebrates lack a similar defense mechanism. Our discovery that **human phagocytes produce an analogous chitinase** is therefore of importance.

DRPR:

Fluorescence in situ hybridization of **human metaphase chromosomes with a genomic chitotriosidase** clone showed that the chitotriosidase gene locus is

1q31-q32.

DRPR:
FIG. 12 shows in situ hybridization to localize mRNA encoding TRAP, osteopontin, **chitotriosidase and HC gp-39 in human** atherosclerotic plaques.

DEPR:
Chitotriosidase is highly homologous to human cartilage glycoprotein of 39 kDa (Hcgp39) [6]. We have demonstrated that Hcgp 39 can be viewed as the lectin counterpart of chitotriosidase [7]. Hcgp39 is produced by chondrocytes and macrophages [5,8] and is thought to play a role as a pathological autoantigen in (rheumatoid) arthritis [9]. Hcgp 39 levels are elevated in synovial fluid of patients with rheumatoid arthritis [8].

DEPR:
5. Boot, R. G., Renkema, G. H., Strijland, A. et al. Cloning of a cDNA encoding **chitotriosidase, a human chitinase** produced by macrophages. J. Biol. Chem. 1995; 270: 26252-26256.

DEPR:
6. Renkema, G. H., Boot, R. G., Muijsers, A. O. et al. Purification and characterization of **human chitotriosidase,** a novel member of the chitinase family of proteins. J. Biol. Chem. 1995; 270: 2198-2202.

DEPR:
7. Renkema, G. H., Boot, R. G., Au, F. L. et al. **Chitotriosidase, a chitinase, and the 39 kDa human** cartilage glycoprotein, a chitin-binding lectin are homologues of family of glycosyl-hydrolases secreted by human macrophages. Eur. J. Biochem. 1998; 251: 504-509.

DEPR:
10. Renkema, G. H., Boot, R. G., Strijland, A. et al. Synthesis, sorting and processing into distinct isoforms of **human macrophage chitotriosidase**. Eur. J. Biochem. 1997; 244: 279-285.

DEPR:
11. Escott, G. M., Adams, D. J. **Chitinase activity in human** serum and leukocytes. Infect. Immun. 1995; 63: 4770-4773.

US-PAT-NO: 6200951
DOCUMENT-IDENTIFIER: US 6200951 B1
TITLE: Chitinase chitin-binding fragments
DATE-ISSUED: March 13, 2001
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Gray; Patrick W.	Seattle	WA	N/A	N/A
Tjoelker; Larry W.	Kirkland	WA	N/A	N/A

US-CL-CURRENT: 514/2,435/183 ,435/196 ,435/209 ,435/7.8 ,530/324 ,530/350
ABSTRACT:

The present invention provides chitin-binding fragments of **human chitinase,** fragment analogs, purified and isolated polynucleotide sequences encoding such fragments and analogs, and materials and methods for the recombinant production of **human chitinase** fragment products which are expected to be useful as in products for detecting chitin, binding chitin, and treating fungal infections or for development of products useful for treating the same.
10 Claims, 0 Drawing figures
Exemplary Claim Number: 1

DATE FILED: March 12, 1998

---------- KWIC ---------

ABPL:
The present invention provides chitin-binding fragments of **human chitinase,** fragment analogs, purified and isolated polynucleotide sequences encoding such fragments and analogs, and materials and methods for the recombinant production of **human chitinase** fragment products which are expected to be useful as in products for detecting chitin, binding chitin, and treating fungal infections or for development of products useful for treating the same.

BSPR:
The present invention relates generally to materials comprising chitin-binding fragments of **human chitinase** enzyme and analogs of the fragments. More particularly, the invention relates to novel purified and isolated polynucleotides encoding such fragment products, to the chitinase fragment products encoded by such polynucleotides, to materials and methods for the recombinant production of such chitinase fragment products and to therapeutic and diagnostic uses of such chitinase fragment products.

BSPR:
Escott et al., Infect. Immun., 63:4770-4773 (1995) demonstrated **chitinase enzymatic activity in human** leukocytes and in human serum. Overdijk et al., Glycobiology, 4:797-803 (1994) described isolation of a chitinase (4-methylumbelliferyl-tetra-N-acetylchitotetraoside hydrolase) from human serum and rat liver. Renkema et al., J. Biol. Chem., 270:2198-2202 (February 1995) prepared a **human chitotriosidase** from the spleen of a Gaucher disease patient. Their preparation exhibited chitinase activity and the article reports a small

amount of amino acid sequence of the protein component of the preparation (22 amino terminal residues and 21 residues of a tryptic fragment). The function of **human chitinase** is also unknown, but a relationship with the pathophysiology of Gaucher disease is proposed in the article. A later publication by the same group [Boot et al., J. Biol. Chem., 270(44):26252-26256 (November 1995)] describes the cloning of a human macrophage cDNA encoding a product that exhibits chitinase activity. The partial amino acid sequence reported by the group in their February 1995 article matches portions of the deduced amino acid sequence of the human macrophage cDNA product. See also International Patent Publication No. WO 96/40940, which reports two distinct **human chitotriosidase** cDNAs encoding a 50 kD and a 39 kD product, both of which were fully enzymatically active. Renkema et al., Eur. J. Biochem., 244:279-285 (1997) reported that **human chitinase** is initially produced in macrophages as a 50 kD protein that is in part processed into a 39 kD form that accumulates in lysozymes, and also reported that alternative splicing generates a distinct **human chitinase** mRNA species encoding a 40 kD chitinase. Both the 39 kD and 40 kD isoforms appeared to be C-terminally truncated and displayed full chitinase enzymatic activity but bound chitin poorly.

BSPR:
The present invention provides novel purified and isolated polynucleotides (i.e., DNA and RNA, both sense and antisense strands) encoding **human chitinase** fragments and analogs thereof having chitin-binding activity but lacking chitinase enzymatic activity; methods for the recombinant production of such fragment products; purified and isolated **human chitinase** polypeptide fragment products; pharmaceutical compositions comprising such fragment products; and diagnostic or therapeutic agents conjugated to such fragment products thereof. Such fragment products and diagnostic or therapeutic agents conjugated thereto are expected to be useful for detecting chitin, binding chitin, and treating fungal infections or for development of products useful for treating fungal infections.

BSPR:
The nucleotide sequence of two human cDNAs encoding presumed allelic variants of **human chitinase,** and including noncoding 5' and 3' sequences, are set forth in SEQ ID NO: 1 and SEQ ID NO: 3. The **human chitinase** coding region corresponds to nucleotides 2 to 1399 of SEQ ID NO: 1 or nucleotides 27 to 1424 of SEQ ID NO: 3, and the putative coding sequence of the mature, secreted **human chitinase** protein without its signal sequence corresponds to nucleotides 65 to 1399 of SEQ ID NO: 1, or nucleotides 90 to 1424 of SEQ ID NO: 3. The amino acid sequences of the polypeptides encoded by the DNA of SEQ ID NOS: 1 and 3 are set forth in SEQ ID NO:2 and SEQ ID NO: 4, respectively. Twenty-one amino-terminal amino acids (positions -21 to -1 of SEQ ID NOS: 2 and 4) comprise a signal peptide that is cleaved to yield the mature **human chitinase** protein (positions 1 to 445 of SEQ ID NOS: 2 and 4). It has been determined that the seventy-two C-terminal residues of **human chitinase** are not critical to chitinase enzymatic activity. Example 5 below illustrates production of an N-terminal fragment that lacks the seventy-two C-terminal residues of **human chitinase**; the introduction of a stop codon after the codon for amino acid 373 resulted in a recombinant chitinase fragment of about 39 kDa that retained similar specific chitinase enzymatic activity when compared with full length recombinant **human chitinase**. The cloning of **human chitinase** cDNA and

expression thereof, and the biological activities of recombinant **human chitinase** are described in detail in U.S. application Ser. No. 08/877,599 filed Jun. 16, 1997, which is a continuation-in-part of U.S. application Ser. No. 08/663,618 filed Jun. 14, 1996, both of which are incorporated herein by reference in their entirety.

BSPR:
The present invention is based on the unexpected discovery that substantially all of the chitin-binding activity of **human chitinase** is contained within the 99 C-terminal amino acid residues of the 445 amino acid enzyme. Specifically provided by the present invention are chitin-binding, chitinase-inactive polypeptide products. Preferred chitinase fragment products comprise a chitin-binding fragment within the 54 C-terminal amino acids of **human chitinase,** including a fragment consisting of about the 99 C-terminal amino acids of **human chitinase** (about residues 347 through 445 of SEQ ID NO: 2) and a fragment consisting of about the 54 C-terminal amino acids of **human chitinase** (about residues 392 through 445 of SEQ ID NO: 2). Also provided by the invention are purified, isolated polynucleotides including DNA encoding such polypeptide fragments; vectors comprising such DNAs, particularly expression vectors wherein the DNA is operatively linked to an expression control DNA sequence; host cells stably transformed or transfected with such DNAs in a manner allowing the expression in said host cell of **human chitinase** fragment products; a method for producing **human chitinase** polypeptide fragment products comprising culturing such host cells in a nutrient medium and isolating such polypeptides from said host cell or said nutrient medium; purified, isolated polypeptides produced by this method; fusion proteins comprising such polypeptides fused to a heterologous polypeptide, including an enzyme such as secreted alkaline phosphatase (SEAP); compositions comprising such human polypeptide fragment products; compositions comprising a **human chitinase** polypeptide fragment product conjugated to an anti-fungal agent and methods of treating fungal infection by administering such compositions; compositions comprising a chitinase polypeptide fragment product conjugated to a detectable label (including radioisotopes, fluorophores, dyes, electron-dense compounds and enzymes), methods for using such compositions to determine the presence or amount of chitin in a sample, comprising the steps of: (a) contacting the sample with a **human chitinase** polypeptide fragment product conjugated to a detectable label, and (b) determining the amount of labelled fragment product bound to chitin, and corresponding kits for diagnosing the presence of chitin in a sample.

BSPR:
Chitinase polypeptide fmgment products of the invention include fragments of **human chitinase** or allelic variants thereof that substantially retain chitin-binding activity without retaining substantial chitinase enzymatic activity, analogs of such fragments, and fusion proteins comprising such fragments or analogs. Chitinase polypeptide fragment products are useful in therapeutic and diagnostic applications as described below.

BSPR:
Among the fragments contemplated by the invention are those represented by amino acid residues X through Y of SEQ ID NO: 2, wherein X is a consecutive integer from 347 through 392 and Y is 445, and portions thereof that retain

chitin-binding activity. One preferred fragment consists of the ninety-nine C-terminal amino acids of **human chitinase** (residues 347 through 445 of SEQ ID NO: 2); this fragment has been shown in Example 7 below to retain 80% of the chitin-binding activity of the mature chitinase. Yet another preferred fragment consists of the fifty-four C-terminal amino acids of **human chitinase** (residues 392 through 445 of SEQ ID NO: 2), which has also been shown to retain chitin-binding activity. As illustrated in Example 7, a fusion protein containing the 99 C-terminal amino acids of **human chitinase** was shown to contain the chitin-binding domain of the protein. The boundaries of the chitin-binding domain were further defined by N-terminal and C-terminal truncation of this 99 amino acid region and determination of the chitin binding activity of fusion proteins comprising these truncates. These truncates included those with an N-terminus commencing at amino acid residue 347, 374. 392, 400 or 409 and with a C-terminus at amino acid residue 431 or 445.

BSPR:
Preferred DNA sequences of the invention include genomic and cDNA sequences as well as wholly or partially chemically synthesized DNA sequences encoding chitin-binding fragments of **human chitinase** without chitinase enzymatic activity, analogs thereof, and fusion proteins comprising such fragments or analogs. Among the nucleotide sequences contemplated by the invention are those encoding the amino acid sequences of positions X through Y of SEQ ID NO: 2, wherein X is a consecutive integer from 347 through 392 and Y is 445. Nucleotides 1238 through 1399 of SEQ ID NO: 1 (encoding residues 392 through 445 of SEQ ID NO: 2) are a particularly preferred DNA sequence of the invention. This DNA sequence and other DNA sequences which hybridize to the noncoding strand thereof under standard stringent conditions or which would hybridize but for the redundancy of the genetic code, and which encode chitin-binding fragments of a chitinase, are also contemplated by the invention. Exemplary stringent hybridization conditions are as follows: hybridization at 42.degree. C. in 50% formamide and washing at 60.degree. C. in 0.1.times.SSC, 0.1% SDS. It is understood by those of skill in the art that variation in these conditions occurs based on the length and GC nucleotide base content of the sequences to be hybridized. Formulas standard in the art are appropriate for determining exact hybridization conditions. See Sambrook et al., 9.47-9.51 in Molecular Cloning, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989).

BSPR:
Among the uses for the polynucleotides of the present invention are use as a hybridization probe, to identify and isolate non-human genomic DNA and cDNA encoding chitin-binding regions of proteins homologous to **human chitinase**; and to identify those cells which express chitin-binding portions of such proteins and the biological conditions under which such proteins are expressed.

BSPR:
In another aspect, the invention includes biological replicas (i.e., copies of isolated DNA sequences made in vivo or in vitro) of DNA sequences of the invention. Autonomously replicating recombinant constructions such as plasmid and viral DNA vectors incorporating polynucleotides encoding chitin-binding fragments of **human chitinase,** including any of the DNAs described above, are provided. Preferred vectors include expression vectors in which the incorporated chitinase fragment-encoding cDNA is operatively linked to an

endogenous or heterologous expression control sequence and a transcription terminator. Such expression vectors may further include polypeptide-encoding DNA sequences operably linked to the chitinase fragment-encoding DNA sequences, which vectors may be expressed to yield a fusion protein comprising the polypeptide of interest.

BSPR:
Knowledge of DNA sequences encoding the chitin-binding portion of **human chitinase** allows for modification of cells to permit or increase expression of the chitin-binding portions. Cells can be modified, (e.g., by homologous recombination) to provide increased expression of the chitin-binding portion of **human chitinase** by inserting all or part of a heterologous promoter in the appropriate position within the gene. The heterologous promoter is inserted in such a manner that it is operably linked to the DNA sequence encoding the chitin-binding portion of **human chitinase**. See, for example, PCT International Publication Nos. WO 94/12650, WO 92/20808 and WO 91/09955. Amplifiable marker DNA and/or intron DNA may be inserted along with the heterologous promoter DNA.

BSPR:
The invention further comprehends use of chitinase fragment products in screening for proteins or other molecules (e.g., small molecules) that specifically bind to the chitin-binding domain of **human chitinase or that modulate binding of human chitinase to chitin or to human** extracellular matrix proteins such as hyaluronic acid. Proteins or other molecules (e.g., small molecules) which specifically bind to chitinase can be identified using fragments of chitinase isolated from plasma, recombinant chitinase fragment products, or cells expressing such products. Proteins or other molecules that bind to the chitin-binding domain of chitinase may be used to modulate its activity. Binding proteins specific for chitinase are contemplated by the invention and include antibody substances (e.g., monoclonal and polyclonal antibodies, single chain antibodies, chimeric antibodies, CDR-grafted antibodies and humanized forms of such antibodies). Binding proteins are useful, in turn, in compositions for immunization as well as for purifying chitinase, and are useful for detection or quantification of chitinase in fluid and tissue samples by known immunological procedures. Anti-idiotypic antibodies specific for chitinase-specific antibody substances are also contemplated.

BSPR:
The scientific value of the information contributed through the disclosures of DNA and amino acid sequences of the present invention is manifest. As one series of examples, knowledge of the sequence of a cDNA for chitinase makes possible the isolation by DNA/DNA hybridization or polymerase chain reaction (PCR) of genomic DNA sequences encoding other mammalian chitinases and the like. DNA/DNA hybridization or PCR procedures carried out with DNA sequences of the invention under conditions of stringency standard in the art are likewise expected to allow the isolation of DNAs encoding **human allelic variants of chitinase, other structurally related human** proteins sharing the chitin-binding property of chitinase, and the chitin-binding regions of non-**human species proteins homologous to chitinase**. The DNA sequence information provided by the present invention also makes possible the development, by homologous recombination or "knockout" strategies [see, e.g.,

Kapecchi, Science, 244: 1288-1292 (1989)], of animals that fail to express a functional chitinase enzyme, overexpress chitinase enzyme, or express a variant chitinase enzyme. Such animals are useful as models for studying the in vivo activity of chitinase or modulators of chitinase. Polynucleotides of the invention when suitably labelled are useful in hybridization assays to detect the capacity of cells to synthesize chitinase. Polynucleotides of the invention may also be the basis for diagnostic methods useful for identifying a genetic alteration(s) in the chitinase locus that underlies a disease state or states. Also made available by the invention are anti-sense polynucleotides relevant to regulating expression of chitinase by those cells which ordinarily express the same.

BSPR:
The **human chitinase** fragment products of the invention are also useful as a chitin-specific reagent for specifically identifying the presence of chitin in a sample. According to this aspect of the invention, a chitinase fragment product having chitin-binding activity is conjugated with a detectable label, such as a radioisotope, fluorophore, dye, electron-dense compound, or enzyme, contacted with the sample to be tested, and analyzed qualitatively or quantitatively for the presence of chitin. "Conjugated" as used herein means linked by covalent bonds.

BSPR:
Such techniques are well known and illustrated in, e.g., U.S. Pat. No. 5,587,292, incorporated herein by reference. The amount of chitin thus measured can be indicative of the fungal load in an infected patient. One preferred fragment for use according to this method is the 54 amino acid chitin-binding domain consisting of amino acid residues 392 through 445 of the **human chitinase** amino acid sequence set out in SEQ ID NO: 2.

BSPR:
Specifically contemplated by the invention are compositions comprising chitinase fragment products for use in methods for treating a mammal susceptible to or suffering from fungal infections. It is contemplated that the chitinase fragment products may be conjugated to other conventional anti-fungal agents, including amphotericin B and the structurally related compounds nystatin and pimaricin; 5-fluorocytosine; azole derivatives such as fluconazole, ketoconazole, clotrimazole, miconazole, econazole, butoconazole, oxiconazole, sulconazole, terconazole, itraconazole and tioconazole; allylamines-thiocarbamates, such as tolnaftate, naftifine and terbinafine; griseofulvin; ciclopirox olamine; haloprogin; undecylenic acid; and benzoic acid. [See, e.g., Goodman & Gilman, The Pharmacological Basis of Therapeutics, 9th ed., McGraw-Hill, N.Y. (1996).] According to this aspect of the invention, the chitin-binding fragment products serve as a vector to target known fungicidal or fungistatic compounds to pathogenic chitin-bearing fungi, and thus may improve the effectiveness of these conventional anti-fungal agents, perhaps by rendering the fungi more susceptible to their action. A reduction in the amount of conventional anti-fungal agent needed to exert the desired therapeutic effect may allow the drugs to be used at less toxic levels. Using **human chitinase** chitin-binding domain for this purpose is more advantageous than using chitin-binding domains of **chitinases of other species because human** polypeptides are expected to be non-immunogenic in humans.

BSPR:
The **human chitinase** cDNA has been isolated from a macrophage cDNA library. Macrophages are known to be closely associated with rheumatoid arthritis lesions [Feldman et al., Cell, 85:307-310 (1996)], and macrophage products such as TNF-.alpha. are implicated in disease progression. A protein with homology to **human chitinase**, C-gp39, has been detected in the synovium and cartilage of rheumatoid arthritis patients. While the natural substrate for **human chitinase** is probably chitin from pathogenic organisms, the enzyme may also exhibit activity on endogenous macromolecules which form the natural extracellular matrix. For example, it has been suggested that hyaluronic acid, a major component of the extracellular matrix, contains a core of chitin oligomers. [Semino el al., Proc. Nat'l Acad. Sci., 93:4548-4553 (1996); Varki, Proc. Nat'l. Acad. Sci., 93:4523-4525 (1996).] Chitinase may therefore be involved in degradation of extracellular matrix in diseases such as rheumatoid arthritis. The role of chitinase may be determined by measuring chitinase levels and/or the effects of chitinase administration or chitinase inhibition in synovial fluid isolated from arthritic joints. Endogenous chitinase levels can be measured by enzymatic assay or with an antibody. Viscosity of synovial fluid can be measured before and after chitinase treatment; a decrease of viscosity associated with chitinase would be consistent with an endogenous chitinase substrate. Modulation of chitinase activity could thereby modulate the progression of joint destruction in rheumatoid arthritis.

BSPR:
Other aspects and advantages of the present invention will be understood upon consideration of the following illustrative examples. Example 1 describes the isolation of **human chitinase cDNA clones from a human** macrophage cDNA library. Example 2 addresses the pattern of **chitinase gene expression in various human** tissues. Example 3 describes the recombinant expression of the **human chitinase** gene in prokaryotic cells and purification of the resulting enzyme. Example 4 provides a protocol for the recombinant production of **human chitinase** in yeast. Example 5 describes the recombinant expression of the **human chitinase** gene in mammalian cells and purification of the resulting protein. Example 6 describes production of **human chitinase** polypeptide analogs and fragments by peptide synthesis or recombinant production methods. Example 7 describes production of **human chitinase** fragments having chitin-binding activity and analogs thereof. Example 8 provides a protocol for generating monoclonal antibodies that are specifically immunoreactive with **human chitinase**. Example 9 describes an assay for the measurement of chitinase catalytic activity. Example 10 addresses determination of the anti-fungal activity of test drugs in vitro. Example 11 addresses determination of the anti-fungal activity of test drugs in vivo in a mouse model, and Examples 12 through 15 address rabbit models of invasive aspergillosis, disseminated candidiasis, Candida ophthalmitis, and Candida endocarditis.

DEPR:
The nucleotide and deduced amino acid sequence of these cDNA clones were compared to sequences in nucleotide and peptide sequence databases to determine similarity to known genes. Sequence comparisons were performed by the BLAST Network Service of the National Center for Biotechnology Information using the alignment algorithm of Altschul et al., J. Mol. Biol., 215:403-410 (1990). Clone MO-911 exhibited significant homology to several different sequences,

including mouse macrophage secretory protein YM-1 precursor (Genbank accession no. M94584), human cartilage gp-39 (Hakala et al., supra), oviductal glycoprotein from sheep, cow, and humans (DeSouza et al., supra), and chitinases from parasite (Oncocerca, Genbank accession no. U14639), wasp (Chelonus, Genbank accession no. U10422), plant (Nicotiana, Genbank accession no. X77111), and bacteria (Serratia, Genbank accession no. Z36295); its highest observed homology was to mammalian genes that encoded proteins with chitinase homology but no demonstrated chitinase activity. Further sequence analysis of MO-911 suggested that it contained a portion of the coding region for a **human chitinase** homolog.

DEPR:
The DNA sequence of clone pMO-218 (deposited on Jun. 7, 1996 under the terms of the Budapest Treaty with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md. 20852, U.S.A. under Accession No. 98077) is set forth in SEQ ID NO: 1, and the encoded amino acid sequence is set forth in SEQ ID NO: 2. MO-218 appeared to include the entire coding region of the **human chitinase** cDNA (nucleotides 2 to 1399 of SEQ ID NO: 1), which comprises a twenty-one amino acid putative signal sequence followed by 445 encoded amino acids (residues 1 to 445 of SEQ ID NO: 2). The twenty-two amino acids following the putative signal sequence exactly match the amino-terminal sequence of purified **human chitotriosidase** reported in Renkema et al., supra. Renkema et al. also described a twenty-one amino acid sequence from a tryptic fragment of **human chitotriosidase** which corresponds exactly to residues 157 to 177 of MO-218 (SEQ ID NO: 2). Boot et al., supra, report the cloning of a **human chitotriosidase** cDNA which contains a coding sequence essentially identical to that of MO-218. The sequence of MO-218 differs from Boot et al. by an additional fourteen nucleotides at the 5' end and by a nucleotide change at nucleotide 330 in the coding region.

DEPR:
Northern blot analysis was performed to identify tissues in which the **human chitinase** is expressed. A multiple human tissue Northern blot (Clontech, Palo Alto Calif.) was hybridized with the entire coding region of MO-218 under standard stringent conditions (according to the Clontech laboratory manual). Greatest hybridization was observed to lung tissue (+++) and ovary (+++), with much smaller levels (+) in thymus and placenta. The size of the hybridizing mRNA was 2.0 kb for lung, ovary and thymus, which corresponds well with the size of the cloned cDNA (1.6 kb, or about 1.8 kb including the polyA tail). The size of the hybridizing placental mRNA was considerably smaller, at 1.3 kb. Chitinase hybridization was not observed in spleen, prostate, testes, small intestine, colon, peripheral blood leukocytes, heart, brain, liver, skeletal muscle, kidney, or pancreas. Chitinase expression in lung is consistent with a protective role against pathogenic organisms that contain chitin, since the lung represents the primary route of entry for fungal pathogens.

DEPR:
Transformants containing the resulting expression plasmid (pAraMO218) were induced with arabinose and grown at 37.degree. C. These transformants produced inclusion bodies containing a 39 kDa protein which was a truncated form of chitinase (engineered to contain 373 instead of 445 amino acids). This chitinase fragment contains four cysteine residues, while the full length

chitinase contains ten cysteine residues. The inclusion bodies were separated from the E. coli culture and electrophoresed on SDS-PAGE. The 39 kDa band was transferred to a PVDF membrane and amino terminal sequenced. The majority (about two-thirds) of the material contained a sequence corresponding to the amino terminus of **human chitinase**. The remaining material corresponded to a contaminating E. coli protein, porin. This recombinant chitinase preparation from E. coli was useful for producing polyclonal and monoclonal antibodies (described below in Example 8).

DEPR:
Exemplary protocols for the recombinant expression of **human chitinase** in yeast and for the purification of the resulting recombinant protein follow. The coding region of **human chitinase** is engineered into vectors for expression in Saccharomyces cerevisiae using either PCR or linker oligonucleotides designed to encode a fusion polypeptide containing a secretion mediating leader to the coding region for **human chitinase** corresponding to the amino terminus of the natural molecule. Secretion signal peptides include, e.g., SUC2 or equivalent leaders with a functional signal peptidase cleavage site, or pre-pro-alpha factor or other complex leader composed of a pre, or signal peptide, and a pro, or spacer region, exhibiting a KEX2 cleavage site. The DNA encoding the signal sequence can be obtained by oligonucleotide synthesis or by PCR. The DNA encoding the pre-pro-alpha factor leader is obtained by PCR using primers containing nucleotides 1 through 20 of the alpha mating factor gene and a primer complementary to nucleotides 255 through 235 of this gene [Kurjan and Herskowitz, Cell, 30:933-943 (1982)]. The pre-pro-alpha leader coding sequence and **human chitinase** coding sequence fragments are ligated into a plasmid containing the yeast alcohol dehydrogenase (ADH2) promoter, such that the promoter directs the expression of a fusion protein. As taught by Rose and Broach, [Meth. Enz., 185:234-279, D. Goeddel, ed., Academic Press, Inc., San Diego, Calif. (1990)], the vector further includes an ADH2 transcription terminator downstream of the cloning site, the yeast "2-micron" replication origin, a selectable marker, for example TRP1, CUP1 or LEU2 (or LEU2-d) or other equivalent gene, the yeast REP1 and REP2 genes, the E. coli beta lactamase gene, and an E. coli origin of replication. The beta-lactamase and TRP1 genes provide for selection in bacteria and yeast, respectively. The REP1 and REP2 genes encode proteins involved in plasmid copy number replication.

DEPR:
The DNA constructs described in the preceding paragraphs are transformed into yeast cells using a known method, e.g. lithium acetate treatment [Stearns et al., Meth. Enz., supra, pp. 280-297] or by equivalent methods. The ADH2 promoter is induced upon exhaustion of glucose in the growth media [Price et al., Gene, 55:287 (1987)]. The pre-pro-alpha sequence or other leader sequence effects secretion of the fusion protein, releasing the mature **human chitinase** peptide from the cells. The signal peptide leader is processed by signal peptidase or, in the case of pre-pro-alpha removal of the pro region, by the KEX2 protease [Bitter et al., Proc. Natl. Acad. Sci. USA, 81:5330-5334 (1984)].

DEPR:
The secreted recombinant **human chitinase** is purified from the yeast growth medium by, e.g., the methods used to purify chitinase from bacterial and mammalian cell supernatants (see Example 3 above and Example 5 below).

DEPR:
The MO-218 clone and the MO-13B clone, both of which contain full length **human chitinase** cDNA 3' to the CMV promoter of pRc/CMV, were isolated. A third plasmid, which corresponded to the same C-terminal fragment expressed in bacterial cells in Example 3 above, was prepared as follows. The MO-218 plasmid was amplified by PCR using oligonucleotide primer 218-1 (CGCAAGCTTGAGAGCTCCGTTCCGCCACATGGTGCGGTCTGTGGCCTGG G, SEQ ID NO: 12), which
contains a Hind III site and nucleotides 2 through 23 of the MO-218 chitinase cDNA of SEQ ID NO: 1, and with complementary downstream primer T-END (GACTCTAGACTAGGTGCCTGAAGGCAAGTATG, SEQ ID NO: 13), which contains nucleotides
1164 through 1183 of MO-218, a stop codon and an Xbal site. The amplified DNA was purified by electrophoresis, digested with Xbal and HindIII, and cloned into the pRc/CMV vector (Invitrogen, San Diego, Calif.) previously cut with the same restriction enzymes. The junctions of the resulting clone was sequenced on a Model 373 (Applied Biosystems, Foster City, Calif.) and encoded the predicted engineered protein sequence, set forth in SEQ ID NO: 14.

DEPR:
Recombinant **human chitinase** was purified as follows. Five days after transfection of COS cells with the pRc/CMV-MO-13B plasmid, conditioned media from the culture was harvested and diluted with an equal volume of water. The diluted conditioned media was applied to a Q-Sepharose Fast Flow column (Pharmacia Biotech, Uppsala, Sweden) which was pre-equilibrated in 25 mM Tris, 10 mM sodium chloride, 1 mM EDTA, at pH 8.0. Approximately 95% of the chitinase activity flowed through and did not bind to the column. This Q-Sepharose flow through was adjusted to 1.2 M ammonium sulfate and applied to a Butyl-Sepharose 4 Fast Flow column (Pharmacia) which was pre-equilibrated in 25 mM Tris, 1.2 M ammonium sulfate, 1 mM EDTA, at pH 8.0. Protein was eluted using a reverse gradient of 1.2 M to 0 M ammonium sulfate in 25 mM Tris, pH 8.0. A single absorbance peak at 280 nm corresponding to the chitinase activity peak was eluted at low salt. This material was greater than 85% pure as determined by SDS-PAGE and contained approximately 60% of the chitinase activity. The protein was then concentrated and buffer exchanged into 20 mM Tris, 150 mM sodium chloride, at pH 8.0 using a 10,000 MWCO membrane (Ultrafree 10K, Millipore Corp., Bedford, Mass.). This preparation was then tested for enzymatic and anti-fungal activity in vitro as described in Examples 9 and 10 below. The recombinant preparation had a chitotriosidase activity of 90 nm/min per mg protein.

DEPR:
The supernatant from the pHDEF1/CTN.1 transfected CHO cells containing overexpressed recombinant **human chitinase** (rH-Chitinase) was purified as follows. In preparation for anion exchange chromatography, the supernatant was diluted 1:3 with 20 mM Tris, pH 7.0 (Buffer A). An anion exchange column, packed with Q-Sepharose Fast Flow Resin (Pharmacia Biotech Inc., Piscataway, N.J.), was equilibrated with Buffer A and loaded with 15L diluted supernatant per 1L resin. The rH-Chitinase was collected in the Flow Through from the Q-Sepharose column and adjusted to 5% Polyethylene Glycol (PEG) 400 (Mallinckrodt Baker, Inc., Phillipsburg, N.J.), 30 mM sodium acetate, pH 4.3 in preparation for cation exchange chromatography. A cation exchange column,

packed with CM-Sepharose Fast Flow Resin (Phamacia Biotech Inc., Piscataway, N.J.), was equilibrated with 30 mM sodium acetate, 5% PEG 400, pH 4.3 (Buffer B). The rH-Chitinase sample was loaded onto the CM-Sepharose column at 1 mg per mL resin, and rH-Chitinase was eluted from the column with 4 mM Tris, 5% PEG 400, pH 7.5 (Buffer C). The rH-Chitinase sample was then prepared for hydrophobic interaction chromatography by adding (NH4).sub.2 SO.sub.4 to 1.5 M. A column packed with Macro-Prep Methyl H1C Support, (Bio-Rad Laboratories, Hercules, Calif.,) was equilibrated with 20 mM Tris, 5% PEG 400, pH 7.0 (Buffer D) containing 1.5M (NH.sub.4).sub.2 SO.sub.4. The rH-Chitinase sample was loaded onto the Macro-Prep Methyl column at 1 mg per mL resin. The column was washed with Buffer D containing 1.1M (NH.sub.4).sub.2 SO.sub.4, and rH-Chitinase was eluted with Buffer D containing 0.2M (NH.sub.4).sub.2 SO.sub.4. The purified eluate was exchanged into 150 mM NaCl, 20 mM Tris, pH 7.0 (Buffer E) by membrane filtration.

DEPR:
Recombinant techniques such as those described in the preceding examples may be used to prepare **human chitinase** polypeptide analogs or fragments. More particularly, polynucleotides encoding **human chitinase** are modified to encode polypeptide analogs of interest using well-known techniques, e.g., site-directed mutagenesis and polymerase chain reaction. C-terminal and N-terminal deletions are also prepared by, e.g., deleting the appropriate portion of the polynucleotide coding sequence. See generally Sambrook et al., supra, Chapter 15. The modified polynucleotides are expressed recombinantly, and the recombinant polypeptide analogs or fragments are purified as described in the preceding examples.

DEPR:
Residues critical for **human chitinase** activity are identified, e.g., by homology to other **chitinases and by substituting alanines for the native human chitinase** amino acid residues. Cysteines are often critical for the functional integrity of proteins because of their capacity to form disulfide bonds and restrict secondary structure. To detennine whether any of the cysteines in **human chitinase** are critical for enzymatic activity, each cysteine is mutated individually to a serine.

DEPR:
A 39 kDa C-terminally truncated fragment of the mature **human chitinase** protein was prepared as described above in Examples 3 and 5 by introduction of a stop codon after the codon for amino acid 373. This 39 kDa fragment lacked seventy-two C-terminal amino acid residues of the mature protein, including six cysteines, yet retained similar specific enzymatic activity compared to the full length recombinant **human chitinase**. This result indicates that the missing seventy-two C-terminal residues, including the six cysteines, are not required for enzymatic activity.

DEPR:
Further C-terminal deletions may be prepared, e.g., by digesting the 3' end of the truncated **human chitinase** coding sequence described in Example 3 with exonuclease III for various amounts of time and then ligating the shortened coding sequence to plasmid DNA encoding stop codons in all three reading frames. N-terminal deletions are prepared in a similar manner by digesting the

5' end of the coding sequence and then ligating the digested fragments into a plasmid containing a promoter sequence and an initiating methionine immediately upstream of the promoter site. These N-terminal deletion analogs or fragments may also be expressed as fusion proteins.

DEPR:
Alternatively, **human chitinase** polypeptide analogs may also be prepared by full or partial chemical peptide synthesis using techniques known in the art. [See, e.g., synthesis of IL-8 in Clark-Lewis el al., J. Biol Chem., 266:23128-34 (1991); synthesis of IL-3 in Clarke-Lewis et al., Science, 231:134-139 (1986); and synthesis by ligation in Dawson et al., Science, 266:776-779 (1994).] Such synthetic methods also allow the selective introduction of novel, unnatural amino acids and other chemical modifications.

DEPR:
The biological activities, including enzymatic, anti-fungal, and extracellular matrix remodeling activities, of the **human chitinase** polypeptide analogs are assayed by art-recognized techniques, such as those described in Examples 9 to 15 below.

DEPR:
The location of the chitin-binding domain of **human chitinase** was determined by generating fusion proteins comprising N-terminally truncated portions of **human chitinase** and testing these products for chitin-binding activity. First, a chimeric protein comprising full length secreted alkaline phosphatase (SEAP) protein (at the N-terminus of the chimeric protein) [Berger et al., Gene, 66:1-10 (1988)] fused to the C-terminal 99 amino acids of **human chitinase** (at the C-terminus of the chimeric protein) was generated as follows. The SEAP component acts as a traceable marker of the chimeric protein.

DEPR:
The SEAP DNA was amplified from the pSEAP2-Control plasmid (Clontech, Palo Alto, Calif.) via polymerase chain reaction (PCR) with primers SEAP Start (SEQ ID NO: 18) and SEAP Stop (SEQ ID NO: 19) that introduced a HindIII site to the 5' end and a multiple cloning region to the 3' end. PCR was carried out using 100 ng of template DNA, 1 .mu.g of each primer, 0.125 mM of each dNTP, 10 mM Tris-HCl, pH 8.4, 50 mM MgCl.sub.2 and 2.5 units of Taq polymerase, with an initial denaturation step of 94.degree. C. for four minutes followed by 30 cycles of amplication: 1 minute at 94.degree. C., 1 minute at 60.degree. C., and 2 minutes at 72.degree. C. This PCR-generated cDNA was cloned into the HindIII and ApaI sites of pcDNA3 (Invitrogen, San Diego, Calif.) to generate a vector called pcDNA-SEAP. DNA encoding the C-terminal 99 amino acids of **human chitinase** (residues 347-445) was also generated by PCR under the same conditions using the primers indicated in Table 1 below, which introduced EcoRI and XhaI sites to the 5' and 3' ends. This PCR-generated chitinase DNA sequence was cloned into the EcoRI and XhaI sites of the multiple cloning region of pcDNA-SEAP.

DEPR:
The resulting construct encoding the chimera was transiently transfected into COS 7 cells by incubation in Dulbecco's modified Eagle medium (DMEM) containing 0.5 mg/ml DEAE dextran, 0.1 mM chloroquine and 10 .mu.g of plasmid DNA for 1.5 hours. The cells then were treated with 10% DMSO in phosphate buffered saline

for 45 seconds, washed with serum-free medium and incubated in DMEM supplemented with 1 mM L-glutamine, 100 U/ml penicillin, 100 .mu.g/ml streptomycin and 10% fetal calf serum. After four days, the culture medium was assayed for SEAP activity as described by Flanagan and Leder, Cell, 63:185-194 (1990). SEAP activity was readily detectable. Incubation of the culture medium containing this fusion protein with insoluble chitin (Sigma, St. Louis, Mo.) for 1 hour at 4.degree. C. resulted in precipitation of more than 80% of the SEAP activity with the chitin. This result demonstrated that the entire chitin-binding domain is contained within the C-terminal 99 amino acids of **human chitinase**.

DEPR:
To determine whether any of the six cysteines within the 99 C-terminal amino acids of **human chitinase** were critical for binding chitin, analogs of chitinase fragments were prepared in which each cysteine was mutated individually to a serine. Six PCR products in which each of the six cysteines was individually mutated to serine were generated using the primers indicated in Table 2 below and fused to SEAP cDNA as described above. Chimeric proteins produced by transiently transfected COS cells were assayed for chitin-binding activity as described above. The results of these experiments demonstrated that each of the six cysteines is required for chitin-binding activity.

DEPR:
A chitin-binding domain fragment consisting of residues 392-445 of SEQ ID NO: 2 was expressed at high levels in the yeast Saccharomyces cerevisiae. An expression construct, .alpha.-FLAG-CBD, was designed in which the nucleotides corresponding residues 392-445 of SEQ ID NO: 2 were fused to the 3' terminus of sequence encoding the S. cerevisiae .alpha.-factor pre-pro sequence [Brake et al., Proc. Natl. Acad. Sci. 81:4642-4646 (1984)] and the FLAG epitope tag (Eastman Kodak). To accomplish this, PCR using primers CBD.alpha.FLAG (sense; SEQ ID NO: 33) and Hu Chit Stop 5 (antisense; SEQ ID NO: 34) was conducted using full-length **human chitinase** DNA as a template. The CBD.alpha.FLAG primer sequence contains an Asp 718 restriction endonuclease site upstream of a FLAG tag-encoding region that is in-frame with the sequence that encodes the first eight amino acids of the chitin-binding domain fragment 392.congruent.445. The Hu Chit Stop 5 primer sequence encodes the C-terminal seven amino acids of the chitin-binding domain fragment followed by Gly-Ala-Gly linked to six histidine residues (His.sub.6) which precede a three amino acid segment prior to the translation termination codon. The His.sub.6 tract is included to facilitate purification of the expressed product by metal affinity chromatography [as described in Nilsson et al., Prot. Expr. Purification 11:1-16 (1997)]. A Not I restriction endonuclease site was included immediately 3' of the stop codon.

DEPR:
The following two protocols (multiple challenge or single shot immunizations) may be used to generate monoclonal antibodies to **human chitinase**. In the first protocol, a mouse is injected periodically with recombinant **human chitinase** (e.g., 10-20 .mu.g emulsified in Freund's Complete Adjuvant) obtained as described in any of Examples 3 through 6. The mouse is given a final pre-fusion boost of **human chitinase** in PBS, and four days later the mouse is sacrificed and its spleen removed. The spleen is placed in 10 ml serum-free RPMI 1640, and a single cell suspension is formed by grinding the spleen between the frosted ends of two glass microscope slides submerged in serum-free

RPMI 1640, supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin, and 100 .mu.g/ml streptomycin (RPMI (Gibco, Canada). The cell suspension is filtered through sterile 70-mesh Nitex cell strainer (Becton Dickinson, Parsippany, N.J.), and is washed twice by centrifuging at 200 g for 5 minutes and resuspending the pellet in 20 ml serum-free RPMI. Splenocytes taken from three naive Balb/c mice are prepared in a similar manner and used as a control. NS-1 myeloma cells, kept in log phase in RPMI with 11% fetal bovine serum (FBS) (Hyclone Laboratories, Inc., Logan, Utah) for three days prior to fusion, are centrifuged at 200 g for 5 minutes, and the pellet is washed twice as described in the foregoing paragraph.

DEPR:
On days 2, 4, and 6, after the fusion, 100 .mu.l of medium is removed from the wells of the fusion plates and replaced with fresh medium. On day 8, the fusion is screened by ELISA, testing for the presence of mouse IgG binding to **human chitinase** as follows. Immulon 4 plates (Dynatech, Cambridge, Mass.) are coated for 2 hours at 37.degree. C. with 100 ng/well of **human chitinase** diluted in 25 mM Tris, pH 7.5. The coating solution is aspirated and 200 ul/well of blocking solution [0.5% fish skin gelatin (Sigma) diluted in CMF-PBS] is added and incubated for 30 min. at 37.degree. C. Plates are washed three times with PBS with 0.05% Tween 20 (PBST) and 50 .mu.l culture supernatant is added. After incubation at 37.degree. C. for 30 minutes, and washing as above, 50 .mu.l of horseradish peroxidase conjugated goat anti-mouse IgG(fc) (Jackson ImmunoResearch, West Grove, Pa.) diluted 1:3500 in PBST is added. Plates are incubated as above, washed four times with PBST, and 100 .mu.L substrate, consisting of 1 mg/ml o-phenylene diamine (Sigma) and 0.1 .mu./ml 30% H.sub.2 O.sub.2 in 100 mM Citrate, pH 4.5, are added. The color reaction is stopped after 5 minutes with the addition of 50 .mu.l of 15% H.sub.2 SO.sub.4. A.sub.490 is read on a plate reader (Dynatech). Selected fusion wells are cloned twice by dilution into 96-well plates and visual scoring of the number of colonies/well after 5 days. The monoclonal antibodies produced by hybridomas are isotyped using the Isostrip system (Boehringer Mannheim, Indianapolis, Ind.).

DEPR:
Alternatively, a second protocol utilizing a single-shot intrasplenic immunization may be conducted generally according to Spitz, Methods Enzymol., 121:33-41 (1986). The spleen of the animal is exposed and injected with recombinant **human chitinase** (e.g., 10-20 .mu.g in PBS at a concentration of about 0.02% to 0.04%, with or without an aluminum adjuvant) obtained as described in any of Examples 3 through 6, after which the spleen is returned to the peritoneal cavity and the animal is stitched closed. Three days later, the mouse is sacrificed and its spleen removed. A spleen cell suspension is prepared, washed twice with RPMI 1640 supplemented with 3% fetal calf serum (FCS), and resuspended in 25 ml of the same medium. Myeloma cells (NS-O) are collected at logarithmic growth phase, washed once and added to the spleen cell suspension in a 50 ml tube, at a ratio of 3:1 or 2:1 (spleen cells:myeloma cells). The mixture is pelleted at about 450 g (1500 rpm), the supernatant aspirated, and the pellet loosened by tapping the tube. Fusion is performed at room temperature by adding 1 ml of polyethylene glycol (PEG) 1500 over 1 minute, with constant stirring. The mixture is incubated for another minute, then 1 ml of warm RPMI (30 to 37.degree. C.) is added over 1 minute followed by 5 ml RPMI over 3 minutes and another 10 ml RPMI over another 3 minutes. The

cell suspension is centrifuged and resuspended in about 200 ml of HAT selective medium consisting of RPMI 1640 supplemented with 100 U/ml penicillin, 100 .mu.g/ml streptomycin, 20% FCS, 100 mM hypoxanthine, 0.4 mM aminopterin and 16 mM thymidine. The cell suspension is dispensed in 1 ml volumes into tissue culture plates and incubated at 37.degree. C. in a humid atmosphere with 5% CO.sub.2 -95 % air for 8 to 10 days. Supernatants are aspirated and the cells are fed with 1 ml HAT medium per well, every 2 to 3 days according to cell growth. Supernatants of confluent wells are screened for specific antibodies and positive wells are cloned.

DEPR:
The **chitotriosidase activity of the recombinant human chitinase** produced in COS cells as described in Example 5A was determined to be 90 nm/min per mg protein. Any of the **human chitinase** fragment products of the present invention can also be tested for chitinase enzymatic activity in this manner.

DEPR:
Conventional anti-fungal agents that have been conjugated to **human chitinase** products of the invention can be tested for inhibition of fungal growth in vitro. The two fungi Candida albicans and Aspergillus fumigatis are serious pathogens for immunocompromised patients. Both Candida and Aspergillus are grown in RPMI growth media at 37.degree. C. to approximately 10,000-50,000 colony forming units (CFU) per ml. Serial dilutions of the test drug are added to cultures, and fungal growth is assessed at 24 hours by turbidity of cultures.

DEPR:
The pharmacokinetics of recombinant **human chitinase** in mice were determined as follows. Female Balb/c mice, 6-8 weeks old, were injected intravenously in the tail vein with 0.5 mg/kg, 5.0 mg/kg and 50 mg/kg recombinant **human chitinase**. For each dose, mice were terminally bled at 0.01, 0.25, 1, 8 and 24 hours after injection (2 animals were used per time point per dosage). Serum samples were then assayed for chitinase activity and concentration. Results are shown in Table 3 below.

DEPC:
Chitinase Gene Expression Pattern in Human Tissues

DEPC:
Production of Recombinant **Human Chitinase** in Bacterial Cells

DEPC:
Production of Recombinant **Human Chitinase** in Yeast Cells

DEPC:
Production of Recombinant **Human Chitinase** in Mammalian cells

DEPC:
Production of **Human Chitinase** Analogs and Fragments

DEPC:
Production of **human chitinase** chitin-binding fragments and analogs thereof

DEPC:
Preparation of Monoclonal Antibodies to **Human Chitinase**

ORPL:
Renkema et al., "**Chitotriosidase, a chitinase, and the 39-kDa human** cartilage glycoprotein, a chitin-binding lectin, are homologues of family 18 glycosyl hydrolases secreted by human macrophages," Eur. J. Biochem., 251:504-509 (Jan., 1998).

ORPL:
Argueso et al., "Effect of the Enzymes **Chitinase and Neuraminidase on the Structure of Human** Ocular Mucus," Investigative Ophthalmology & Visual Science, 36(4):S997 (Mar. 15, 1995) (Abstract 4615-596).

ORPL:
Boot et al., "Cloning of cDNA Encoding **Chitotriosidase, A Human Chitinase** Produced by Macrophage," J. Biol. Chem., 270(44):26252-26256 (Nov. 3, 1995).

ORPL:
Escott et al., "**Chitinase Activity in Human** Serum and Leukocytes," Infect. Immun.,63(12):4770-4773 (Dec., 1995).

ORPL:
Overdijk et al., "**Human Serum Contains a Chitinase:** Identification of an Enzyme, Formerly Described as 4-Methylumbelliferyl-tetra-N-Acetylchitotetraoside Hydrolase (MU-TACT Hydrolase)," Glycobiology, 4(6):797-803 (1994).

ORPL:
Renkema et al., "Synthesis, sorting, and processing into distinct isoforms of **human macrophage chitotriosidase,**" Eur. J. Biochem., 244(2):279-285 (1997).

ORPL:
Renkema et al., "Purification and Characterization of **Human Chitotriosidase,** a Novel Member of the Chitinase Family of Proteins," J. Biol. Chem., 270(5):2198-2202 (Feb. 3, 1995).

US-PAT-NO: 6184027
DOCUMENT-IDENTIFIER: US 6184027 B1
TITLE: Isolation and purification of eubacteria and fungus with catalytically inactive murein binding enzymes
DATE-ISSUED: February 6, 2001
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Laine; Roger A.	Baton Rouge	LA	N/A	N/A
Lo; Wai Chun.Jennifer	Baton Rouge	LA	N/A	N/A

US-CL-CURRENT: 435/261
ABSTRACT:
Catalytically inactive murein binding enzyme diagnostic reagents and methods and kits for detecting eubacteria and fungus in biological samples.
8 Claims, 30 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 11

DATE FILED: March 4, 1999

---------- KWIC ---------

DEPU:
7. Escott, G. M. and Adams, D. J. 1995. **Chitinase activity in human** serum and leukocytes. Inf. Imm. 63: 4770-4773.

US-PAT-NO: 6177447
DOCUMENT-IDENTIFIER: US 6177447 B1
TITLE: Deoxynojirimycin derivatives and their uses as glucosylceramidase inhibitors
DATE-ISSUED: January 23, 2001
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Aerts; Johannes Maria F. G.	Abcoude Amsterdam	N/A N/A	N/A N/A	NLX NLX
Pandit; Upendra Kumar	Heiloo	N/A	N/A	NLX
Koomen; Gerrit-Jan	Leiden	N/A	N/A	NLX
Overkleeft; Herman Steven	San Bovio	N/A	N/A	ITX
Vianello; Paola				

US-CL-CURRENT: 514/319,546/195
ABSTRACT:
Deoxynojirimycin derivatives containing a large hydrophobic moiety, such as cholesterol or adamantame-methanol, linked through a spacer, such as pentamethylene, to the nitrogen atom of deoxynojirimycin, and salts thereof, inhibit glucosylceramidase and may be useful in the treatment of diseases involving a ceramide-mediated signaling process, such as Gaucher disease.
7 Claims, 4 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 3

DATE FILED: April 30, 1999

---------- KWIC ---------

BSPR:
Recently a sensitive marker for Gaucher cells has been discovered by us [4]. Using the technique of in situ hybridization we observed that Gaucher cells synthesize large quantities of the secretory enzyme **chitotriosidase, the human analogue of chitinases** present in various species. This explains the dramatic elevation in plasma chitotriosidase levels in clinically affected Gaucher patients. On the average chitotriosidase levels are about 1000 fold higher in plasma of these patients as compared to corresponding normal subjects. In presymptomatic or asymptomatic individuals with an inherited glucocerebrosidase deficiency plasma chitotriosidase levels are (almost) within the normal range (see Table 2). Interestingly, elevated levels of plasma chitotriosidase have also been noted for patients with other sphingolipidoses, in particular Niemann-Pick disease [5].

DEPU:
6. Renkema, G. H., Boot, R. G., Muysers, A. O., Donker-Koopman, W. E., Aerts, J. M. F. G. (1995) J. Biol. Chem. 270, 2198-2202. Purification and characterization of **human chitotriosidase,** a novel member of the chitinase family of proteins.

DEPU:

7. Boot, R. G., Renkema, G. H., Strijland, A. H., van Zonneveld, A. J., Aerts, J. M. F. G. (1995) J. Biol. Chem. 270, 26252-26256. Cloning of a cDNA encoding **chitotriosidase, a human chitinase** produced by macrophages.

US-PAT-NO: 6159719
DOCUMENT-IDENTIFIER: US 6159719 A
TITLE: Pan-bacterial and pan-fungal identification reagents and methods of use thereof
DATE-ISSUED: December 12, 2000
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Laine; Roger A.	Baton Rouge	LA	N/A	N/A
Jennifer Lo; Wai Chun	Baton Rouge	LA	N/A	N/A

US-CL-CURRENT: 435/206,435/18 ,435/7.1
ABSTRACT:
Murein binding polypeptide and antibiotic diagnostic reagents, methods and kits for detecting eubacteria and fungus in biological samples.
10 Claims, 31 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 11

DATE FILED: March 3, 1999

---------- KWIC ---------

DEPU:
7. Escott, G. M. and Adams, D. J. 1995. **Chitinase activity in human** serum and leukocytes. Inf. Imm. 63: 4770-4773.